(51) International Patent Classification[7]: C12N 15/62, 15/63, 5/10, C07K 14/705, A61K 38/17

(21) International Application Number: PCT/US01/29873

(22) International Filing Date:
24 September 2001 (24.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/234,843   22 September 2000 (22.09.2000)   US

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-ance Notes on Codes and Abbreviations" appearing at the begin-ning of each regular issue of the PCT Gazette.

WO 02/24929 A2

(54) Title: A SOLUBLE BETA 2 MICROGLOBULIN (β2M)/HFE MONOCHAIN FOR BIOTECHNOLOGICAL AND THERA-PEUTIC APPLICATIONS

(57) Abstract: A DNA construct encodes a monochain form of beta-2 microglobulin/HFE protein. Particularly, a genetically engi-neered DNA construct encompasses human beta-2 microglobulin (β2m) gene linked via an adjustable linker to specific domains of the human HFE gene, in order to produce a properly conformed, soluble, stable and functional protein. The construct may be used as a platform for drug delivery of the therapeutics of cancer, autoimmune diseases, inflammatory conditions and the like. It can also be used for the therapeutics of iron-overload conditions, oxidative stress conditions and chronic infections.

A SOLUBLE BETA 2 MICROGLOBULIN (β2M)/HFE MONOCHAIN FOR
BIOTECHNOLOGICAL AND THERAPEUTIC APPLICATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S.
provisional application 60/234,843, filed September 22, 2000,
the entire contents of which being hereby incorporated herein
by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention relates to methods for
targeting and delivering of drugs into human cells including
without limitation, tumors and metastasizing cells, activated
lymphocytes operative in autoimmune and inflammatory
conditions, and the like. This invention also relates to
therapeutic interventions for iron overload disorders and
chronic infections.

Background of the Technology

[0003] Millions of people suffer from cancer and autoimmune
diseases all over the world. Currently, most cancer and some
autoimmune diseases remain incurable, and treatments provide
the means to prolong life expectancy and enhance qualities of
life of the sick but do not provide remedy. Current therapies
are facing major problems, particularly:

        1. Lack of specificity. Chemotherapy, radiotherapy
and other anti-cancer therapies damage healthy tissues and
consequently, induce severe side effects.

        2. Development of resistance to anti-cancer
therapies and specifically, to chemotherapy, a phenomena known
as the multi-drug resistance.

[0004] One approach to attack these problems is to target
and modulate the major cellular iron (Fe) transport system, the
transferrin receptor (TfR). Since iron is indispensable for
cellular proliferation, it is excessively uptaken, via the TfR,

by tumors, metastases and activated autoimmune lymphocytes as
well as lymphocytes and leukocytes active in inflammatory
disorders and graft rejection (Ragheb et al 1999, Bayer et al,
1999).

[0005] Previous approaches that manipulated the cellular
iron uptake system for cancer therapy included the following:

         1. Iron supply to tumor cells may be blocked, thus,
leading to growth arrest and death (Kemp, 1997). This was
achieved by utilizing gallium nitrate, a chemical structurally
resembling iron that competes with intracellular iron uptake
(Apseloff et al, 1997, Collery et al, 2000, Webster et al,
2000). However, this chemical is not specific for TfR and
therefore interferes with various iron uptake systems in the
body and causes severe side effects.

         2. Drugs may be delivered into cells through the
internalization of the TfR (Singh, 1999, Hagihara et al, 2000,
Hall, 2000). This was achieved by conjugation of drugs to
transferrin, the natural ligand of TfR. Although transferrin
conjugated to doxorubicin could overcome multi drug resistance
in a cancer cell line (Wang et al, 2000), the administration of
transferrin-conjugates is not an optimal approach since
transferrin stimulates growth, which is contradictory to the
purpose of the treatment. Moreover, it is diluted in the blood
due to high levels of serum transferrin.

         3. Anti-TfR antibodies may be used for drug delivery
into cells (Taetle et al, 1987, White, et al, 1990, Kemp et al,
1992, Brooks et al, 1995, Wening et al, 1999). Usage of these
antibodies has some drawbacks since they do not dissociate from
the receptor inside the cells but recycle back to the cell
surface. Thus, the drug-conjugated antibodies may exert a
cytotoxic effect on surrounding cells and tissues. In
addition, the antibodies developed thus far provoke immune
responses, thus, preventing multiple treatments.

[0006] HFE is a central regulator of iron absorption from the diet and iron uptake by most cells in the body. A recessive autosomal mutation (C282Y) in the HFE gene causes an iron overload disorder (Hereditary Hemochromatosis, HH) common in populations of Northwest European ancestry (Feder et al, 1996; Beutler et al, 1996; Jazwinska et al, 1996; Jouanolle et al, 1996; Barton et al, 1997; Borot et al, 1997; Carella et al, 1997; Worwood, 1997). HH patients accumulate iron first in the transferrin pool and later in parenchymal tissue of many organs leading to multi-organ dysfunction including diverse pathologies of the liver (cirrhosis), pancreas (diabetes), joints (arthritis), heart (arrhythmia), and hypophysis (hypogonadism). Liver damage, including hepatocellular carcinoma, is the major cause of mortality in HH (Niederau et al, 1999).

[0007] Positional cloning of the HFE gene from an HLA-linked region of chromosome 6p (detailed in a review by Feder, 1999) and the subsequent crystallization of the encoded protein (Lebron et al, 1998) revealed significant sequence and structural homology to MHC class I proteins (37% identity in the ectodomain). The gene encodes a 348-residue type I transmembrane glycoprotein, which associates with the class I light chain β2m, and is mutated in most patients with HH (Feder et al, 1996; and U.S. patent 6,140,305). In both HFE and classical class I molecules, the α1-α2 superdomain forms a platform comprising an eight strand antiparallel β-sheet topped by two helices, which is positioned on top of immunoglobulin constant-like domain α3 and β2m. A mutation in the HFE gene is the primary risk factor for the HH disease. 75-90% of affected individuals with clinical HH are homozygous for a position C282Y mutation (Feder et al, 1996). This mutation prevents the formation of a critical disulfide bond in the HFE α3 domain, impairing its ability to form its obligate heterodimer with β2m and, thus, inhibits its delivery to the cell surface (Feder et

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al, 1998). Mice deficient in β2m and mice deficient in HFE or
mice engineered to express the disease-associated C282Y HFE
mutation demonstrate systemic iron overload with iron
deposition in liver parenchymal cells (de Sousa et al, 1994,
5   Rothenberg et al, 1996, Zhou et al, 1998, Bahram et al, 1999,
Levy et al, 2000). Since it was demonstrated that β2m
heterodimerizes with HFE and mice lacking β2m develop iron
overload, it was suggested that HFE requires β2m to exert its
regulatory effect on iron metabolism. Moreover, it was
10   demonstrated that mice lacking both the HFE and the β2m genes
develop more severe iron overload symptoms than mice lacking
HFE alone, suggesting that there may be another, still
unidentified, molecule that interacts with β2m and regulates
intestinal iron absorption. Thus, β2m may play an active role
15   in iron regulation (Levy et al, 2000).

[0008] The interaction of HFE with TfR was demonstrated
biochemically by co-immunoprecipitation of HFE with TfR in
intestinal cells, placental cells, and HFE-transfected cells
(Parkkila et al, 1997; Feder et al, 1998; Gross et al, 1998;
20   Waheed et al, 1999; Salter-Cid et al, 1999). The results were
further supported by the co-localization of HFE and TfR on the
cell surface and in intracellular organelles (Gross et al,
1998; Feder et al, 1998).

[0009] HFE binds TfR tightly at the pH of the cell surface,
25   but not at pH 6, suggesting that HFE is internalized with TfR
and dissociates from the receptor in acidified endosomes
(Lebron et al, 1998; Lebron et al, 1999). Biochemical studies
showed that HFE and ferrum bound-transferrin (Fe-Tf) can bind
simultaneously to TfR to form a ternary complex consisting of
30   one Fe-Tf and one HFE bound to a TfR homodimer (Lebron et al,
1999), and that HFE inhibits the TfR-Tf interaction (Feder et
al, 1998; Gross et al, 1998) by binding near or at the Tf-
binding site of TfR (Lebron et al, 1999). The crystal
structure of a complex between the ectodomains of HFE and TfR

(Bennet et al, 2000) allowed the localization of the TfR
binding site on HFE to the C-terminal portion of the $\alpha1$ domain
helix and an adjacent loop, a region distinct from the ligand-
binding site of a classical class I MHC molecule (Lebron et al,
5   1999).

[0010] HFE-overexpressing cells demonstrate an iron-
deficiency phenotype; they have decreased ferritin expression
(Gross et al, 1998; Roy et al, 1999; Corsi et al, 1999),
modestly increased number or TfRs (Roy et al, 1999), and
10   induced IRP levels (Corsi et al, 1999; Riedel et al, 1999), all
implying low intracellular iron pools.

[0011] Immunohistochemical analyses demonstrated co-
localization of HFE and TfR in intracellular compartments and
on the cell surface (Gross et al, 1998; Feder et al, 1998).
15   Further studies of HFE expression in HFE-GFP-transfected
hepatoma cell line, showed that HFE co-localizes with TfR
mainly in perinuclear regions (Ikuta et al, 2000). The
compiled data suggest that TfR-bound HFE is internalized with
the receptor and is released within the endosomes.

20   [0012] Several research groups have engaged the problem of
growth arrest and cell death in HFE-transfected HeLa cells.
This problem could be solved only when the HFE gene was
expressed under the control of an inducible expression system
(Roy et al 2000, Riedel et al, 1999).

25   SUMMARY OF THE INVENTION

[0013] Since $\beta2m$ appears to play an active role in iron
regulation and since proper folding is crucial for function of
all MHC class I molecules, including HFE, the present inventors
have designed a chimeric monochain protein that maximizes the
30   chances of an HFE-derived polypeptide to associate with its
natural counterpart(s) and to gain full conformation and
function. This was achieved by linking the two polypeptides

(β2m and specific domains of HFE) via an adjustable linker that brings β2m and HFE-derived polypeptides to appropriate folding.

[0014] The monochain is soluble, stable in serum, and functional, as assessed by its binding to TfR. Consequently, it is optimal for the dual designed functions: (a) drug delivery into target cells, and (b) regulation of intracellular iron uptake.

[0015] In particular, this monochain manifests specific characteristics that are advantageous for drug delivery systems:

      1. It is a soluble, stable and fully conformed protein.

      2. It binds specifically to TfR and therefore targets cells that over-express this receptor.

      3. It is continuously internalized by the target cells, thus enabling efficient drug delivery.

      4. It dissociates from the receptor in the cells, thus minimizing side effects.

      5. It negatively regulates iron absorption, thus reducing growth of undesired cells and preventing lymphocyte activation.

      6. It is not diluted in the blood as is transferrin.

      7. It should not induce immune responses since it is a self non-polymorphic protein.

      8. Delivery of drugs via this monochain is expected to overcome drug-resistance since it is a natural TfR-binding protein.

[0016] The monochain will serve as a core for the construction of a drug delivery platform where various chemicals, proteins, peptides, DNA or oligonucleotides are conjugated directly to the protein or indirectly via liposomes or polymer-modified liposomes, as has been previously shown for other TfR-targeting vehicles (Zhang et al, 2001, Kono et al, 2001, Bickel et al, 2001).

[0017] Of particular interest is the option of drug delivery to the brain since the blood-brain barrier (BBB) prevents the penetration of most drugs. An exception is the transferrin molecule that, following its binding to TfR on epithelial cells of the BBB, is internalized and reaches brain cells (Bickel et al, 2001). Therefore, any delivery system utilizing the TfR as a target, shares this advantageous property that is crucial in situations of brain metastases, particularly following the development of drug resistance as in the case of Doxil®, a novel form of doxorubicin in polyethylene-glycol coated liposomes (Lyass, et al, 2000). Therapy that combines chemotherapy and TfR-targeting by the monochain is expected to overcome drug resistance and to enable penetration through the BBB, providing a better and more efficient therapeutic solution for advanced stages of cancer.

[0018] This platform may be expanded into a larger three-polypeptide construct that will deliver a variety of proteins, including regulatory components such as anti-oncogenes for the intervention with cancer progression, and proteins that are mutated in hereditary diseases such as the glucocerebrosidase, which is currently administrated to Gaucher patients.

[0019] Since TfR is over-expressed in activated lymphocytes, a monochain-based drug delivery system should be used in cases that involve activated lymphocytes and leukocytes including graft rejection, skin inflammation, autoimmune disorders and the like.

[0020] The monochain molecule could be also utilized as a biotechnological tool for the insertion of desired DNA or proteins into cells. For example, poor transfection efficiency is the major drawback of lipofection (Yanagihara et al, 2000). The addition of transferrin to liposomes enhances the delivery of DNA, oligonucleotides, and peptides into the cells, in vitro and in vivo (Ogris et al 1999). It is conceivable that the addition of the monochain or its derivatives to liposomes will

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improve their intracellular delivery and may serve as a
possible strategy for gene therapy.

[0021] Since the monochain reaches the endosomes following
its internalization and endosomes serve as a major site for
5   protein cleavage and production of peptides for antigen
presentation to regulatory lymphocytes (T helper cells), the
monochain can be used as a delivery vehicle for antigens into
cells, resulting in the desired antigen presentation to
lymphocytes (Ali et al, 1999, Fernandes et al, 2000). In this
10   way, one could enhance and amplify immune responses and improve
vaccination strategies.

[0022] Soluble β2m/HFE monochain protein provides a
therapeutic solution for treatment of various iron overload
conditions, including, without limitation, hereditary
15   hemochromatosis, and secondary iron overload disorders such as
transfusions, thalassemias, hemolytic anemias and chronic
infections. The β2m/HFE monochain and derivatives also provide
a therapeutic solution to oxidative stress disorders resulting
in tissue damage, including, without limitation, vascular
20   diseases, inflammation, atherosclerosis, lung injury, ischemia
and the like.

[0023] Monochain based therapeutics are to be administered
externally as an ointment, internally directly into the
circulation in perfusion, or orally as tablets, depending on
25   the drug delivered and the disease.

[0024] Thus, according to the present invention, a DNA
construct encoding a soluble monochain β2m/HFE chimera is
provided. The expressed protein serves for delivery of
therapeutic agents into cells by administering an effective
30   dose of monochain conjugated to a therapeutic agent. The
expressed monochain also serves for the regulation of iron
absorption and metabolism in target cells.

[0025] The present invention is also directed to the use of
HFE alone, or an analog, fragment, functional derivative or

salt thereof, for the treatment of iron overload diseases, such
as hereditary hemochromatosis (HH), as well as secondary iron
overload diseases, such as transfusions, thalassemias,
hemolytic anemias, oxidative stress conditions and chronic
5   infections.

[0026] The present invention further relates to the use of
β2m *per se*, its analogs, active fragments, functional
derivatives and salts for the treatment of iron overload
conditions.

10   BRIEF DESCRIPTION OF THE DRAWINGS

[0027] Figures 1a and 1b are radiolabeled nitrocellulose
membrane blots showing that human tumor cell lines express high
levels of TfR and low levels of HFE. Fig. 1c is a table
summarizing the results. Cytoplasmic RNA was extracted from
15   human tumor cell lines, fractionated on agarose gel, blotted on
nitrocellulose membrane and hybridized with the relevant P^{32}-
labeled probes. The following radiolabeled fragments were
used: TfR cDNA, HFE cDNA and H-2K^b cDNA. The membrane was
exposed for 4h to X-ray film following hybridization with TfR
20   and for 2 weeks following hybridization with HFE probes. The
membranes thus obtained are shown in Figs. 1a and 1b. The
signals were scanned by phosphorimager, quantitated and the
relative strength is summarized in the table of Fig. 1c.

[0028] Figs 1d-1k are graphs showing that human tumor cell
25   lines express high levels of surface TfR. Tumor cell lines of
lymphoid origin (K562 (Fig. 1d), CRHF (Fig. 1e), Dami (Fig.
1f), MegO1 (Fig. 1g)) breast carcinoma (T47D (Fig. 1h))
cervical carcinoma (SiHa (Fig. 1i)), and melanoma (GTBS (Fig.
1j), WUBI (Fig. 1k)) were analyzed by FACS for the expression
30   of surface TfR using mAb V1-10. Mean fluorescence values of
each cell line are included above each graph.

[0029] Fig. 2 is the DNA (SEQ ID NO:1) and amino acid (SEQ
ID NO:2) sequence of the β2m/HFE monochain with a C-terminal

myc-tag, followed by a His-tag. The DNA sequence analysis was
performed in the sequencing facilities of Tel Aviv University.
Amino acid translation was performed by the DNA Strider
program.

5   [0030] Fig. 3 is a membrane showing that HEK293/β2m/HFE/Mhm
cells express and secrete β2m/HFE monochain. Individual
HEK293/β2m/HFE/Mhm clones M1-M2.5 were tested for expression
and secretion of β2m/HFE monochain. Cell extracts and
conditioned media were harvested, fractionated on SDS-PAGE,
10   blotted onto nitrocellulose membrane, hybridized with anti-myc
mAb and appropriate horseradish peroxidase-conjugated secondary
antibodies. Immune detection was performed using an ECL kit
according to the manufacturer's directions (Amersham Pharmacia
Biotech).

15   [0031] Fig. 4 is a gel showing that β2m/HFE monochain binds
TfR. HEK293 cells, HEK293/HFE, HEK293/β2m/HFE/Mh and
HEK293/β2m/HFE/Mhm cells were metabolically labeled for 1h.
The cells were harvested and cell extracts were
immunoprecipitated using anti-HFE antibody, 2F5, anti-hβ2m
20   antibody, B2.62.2, and anti-hTFR antibody, V1-10 as indicated.
The immunoprecipitates were fractionated on SDS-PAGE. The gel
was mounted, dried and exposed to X-ray film. Proteins
corresponding to 90 kD (TfR), 55 kD (monochain) 45 kD (HFE
and/or class I MHC) and 12 kD (β2m) are marked with arrows.

25   [0032] Figs. 5a and 5b are gels showing synthesis and
intracellular trafficking studies of HFE and β2m/HFE proteins.
In Fig. 5a, HEK293/β2m/HFE/Mhm cells were metabolically labeled
for 30 minutes and chased as indicated. The cells and
conditioned media were harvested. Cell extracts were
30   immunoprecipitated using anti-HFE antibody, 2F5, and anti-hTfR
antibody, V1-10. The immunoprecipitates and the media (sup.)
were fractionated on SDS-PAGE. The gel was mounted, dried and
exposed to X-ray film. In Fig. 5b, HEK293/HFE cells were
metabolically labeled for 30 minutes and chased as indicated.

Cells were harvested and cell extracts were immunoprecipitated
using anti-TfR antibody, V1-10. The immunoprecipitates were
fractionated on SDS-PAGE. The gel was mounted, dried and
exposed to X-ray film. Proteins corresponding to 90 kD (TfR),
5   and 45 kD (HFE) are marked with arrows.

[0033] Figs. 6a and 6b are gels showing that HeLa cells
cannot over-express HFE-derived molecules. In Fig. 6a, HeLa,
HeLa/HFE, HeLa/HFE(mut.C282Y) and HeLa/β2m/HFE/Mhm cell lines
were tested by RT-PCR for the expression of the transfected
10   genes, using primers corresponding to the α1-α2 domains of HFE
and actin, respectively. Non-specific bands are marked with
stars. In Fig. 6b, HeLa/HFE and HeLa/β2m/HFE/Mhm cell lines
were metabolically labeled for 1 hour, lysed and
immunoprecipitated with anti-HFE antibody, 2F5 and anti-hTFR
15   antibody, V1-10. The immunoprecipitates were separated on SDS-
PAGE. The gel was mounted, dried and exposed to X-ray films.
Proteins corresponding to 55 kD (β2m/HFE monochain) are marked
with arrows. Non-specific bands are marked with stars.

[0034] Figs. 7a and 7b are gels showing that radioactive
20   labeled β2m/HFE/Mhm monochain binds TfR and penetrates into
cells. Human tumor cell lines (HeLa and FO1) and mouse cell
lines (VAD12.79, VAD12.79/hTfR, B16) were incubated, with [35S]-
labeled β2m/HFE/Mhm conditioned media. The cells were
harvested and cell extracts were immunoprecipitated with anti-
25   hTfR antibody V1-10, (Fig. 7a) and anti-HFE antibody 2F5 (Fig.
7b) as indicated. The precipitates were fractionated on SDS-
PAGE. The gel was mounted, dried and exposed to X-ray film.

[0035] Fig. 8 is a gel showing purification of β2m/HFE/Mhm
from conditioned media. β2m/HFE/Mhm proteins were purified as
30   described in the "Specific Techniques" section. Proteins from
different purification steps were fractionated onto SDS-PAGE.
The gel was divided: half was stained with Coomassie for the
estimation of protein concentration (Fig. 8, upper part) and
half was blotted to nitrocellulose membrane and hybridized with

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anti-myc antibodies followed by horseradish peroxidase-
conjugated secondary antibodies (Fig. 8, lower part). Immune
detection was performed using an ECL kit according to the
manufacturer's directions (Amersham Pharmacia Biotech). S-
starting material, FT-flowthrough, W-wash, E-elution. Non-
specific bands are marked with stars.

[0036] Fig. 9 shows membranes demonstrating that externally
added β2m/HFE/Mhm reduces cellular TfR levels. HeLa cells were
incubated for 24 hours with 20 µg/ml purified β2m/HFE/Mhm or
treated with 100 µM DFO. The cells were harvested and cell
extracts were fractionated on SDS-PAGE, blotted to
nitrocellulose membrane and hybridized with anti-hTFR antibody,
V1-10 (upper gel), and anti MAP-kinase antibodies (lower gel)
followed by the appropriate horseradish peroxidase-conjugated
secondary antibodies. Immune detection was performed using an
ECL kit according to the manufacturer's directions (Amersham
Pharmacia Biotech).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0037] Generally, the present invention provides a construct
encoding a soluble β2m/HFE monochain or a functional derivative
or a salt thereof. Preferably, the protein encompasses the
human β2m or an analog or active fragment thereof, an
artificial linker that preferably encodes 15 amino acids, and
the α1-α3 domains of the human HFE protein or an analog or
active fragment thereof. The protein is capable of binding
TfR, being internalized by cells, and regulating iron
metabolism. This protein, when conjugated to a desired agent,
is capable of targeting specific cells and delivering drugs
into cells.

[0038] The term "monochain" as used herein is meant to
include, but is not limited to, recombinant polypeptides that
are linked via a compatible linker to provide a single
recombinant protein.

[0039] The term "soluble" as used here is meant to include, but is not limited to, recombinant proteins or polypeptides that are not directly anchored in cellular membranes.

[0040] The term "therapeutic" as used herein is meant to
5   include, but is not limited to, toxic agents, chemotherapeutic drugs, peptides, polypeptides, proteins, chemical compounds, other desired therapeutics or DNA, or any molecular marker that can be attached to a soluble monochain β2m/HFE protein.

[0041] As indicated above, the soluble β2m/HFE monochain is
10 composed of three parts. The first part is human β2-microglobulin (β2m) whose sequence is known and available at GenBank Accession Nos. NP_004039 and XP_032402. The full sequence of β2m appears as residues 1-119 of SEQ ID NO:2. It should be understood that analogs and active fragments of this
15 sequence can be substituted for the β2m portion of the monochain in accordance with the present invention. The analog or active fragment must retain the properties of β2m that permit the bioactivity of the monochain of the present invention as disclosed herein, i.e., the β2m portion must
20 confer stability and property conformation required for trafficking and function of the formed monochain iron regulation.

[0042] The HFE portion of the monochain is, preferably, the α1-α3 domains of the human HFE protein, i.e., amino acids 134-
25 409 of SEQ ID NO:2. Again, analogs and functional derivatives of this sequence may be substituted, as long as the resulting polypeptide interacts with the β2m portion of the monochain so as to permit the formed monochain to bind TfR and to regulate of iron metabolism in target cells.

30 [0043] The linker portion must be sufficiently flexible to allow the active complex to be maintained. The preferred flexible linker is made of GGGGS (SEQ ID NO:15) repeats. The optimum number of repeats has been determined to be three, although any number of repeats from 1 to 5 may be employed in

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the monochain. Other flexible linkers, as are well known in
the art, may be substituted for the linker described herein as
the preferred embodiment. Such a flexible linker, is
preferably, about 15 amino acids in length, although linkers of
5   from 5 to 25 amino acids can be devised as long as they do not
form a particularly immunogenic epitope between the β2m and the
HFE portions of the monochain and as long as they do not
inhibit the activity of the monochain as disclosed herein.
Preferably, such a linker will have a neutral charge so as not
10   to affect the conformation of the monochain.

    [0044] The flexible linker may have an intracellular
protease cleavage site. Alternatively, an intracellular
protease cleavage site may be located adjacent to the linker.
A variety of proteases are released by necrosing and apoptosing
15   cells. Included in these are caspases (Interleukin 1 beta-
converting enzyme-like proteases), metallo proteinases,
lysosomal proteases (e.g., cathepsin B) and elastase. Elastase
is released by granulocytes during disease states (e.g.,
sepsis) and has a broad specificity regarding amino acid
20   cleavage sequence, akin to that of trypsin (Ertel et al, 1994;
Szilagyi et al, 1995).

    [0045] Intracellular proteases can act on the soluble
β2m/HFE monochain containing an intracellular protease
recognition sequence in the linker portion to allow the β2m and
25   the HFE portions to be separated once delivered into the cell
in order to enhance, to stabilize or to promote other functions
of the monochain associated with iron metabolism.

    [0046] An analog of β2m or HFE as described herein has an
amino acid sequence essentially corresponding to that of the
30   native human β2m or the specified domains of native HFE. The
term "essentially corresponding to" is intended to comprehend
analogs with minor changes to the native sequence of the
specified polypeptide, which changes do not affect the basic
characteristics thereof, particularly insofar as its ability to

be used in the monochain of the present invention, which
monochain binds to TfR and regulates iron absorption. The type
of changes which are generally considered to fall within the
"essentially corresponding to" language are those which would
5 result from conventional mutagenesis techniques of the DNA
encoding the β2M or HFE, resulting in a few minor
modifications, and screening for the desired activity in the
manner discussed above.

[0047] Preferably, the analog is a variant of the native
10 sequence, which variant has an amino acid sequence having at
least 70% identity to the native amino acid sequences and
retains the biological activity thereof. More preferably, such
a sequence has at least 85% identity, at least 90% identity, or
most preferably at least 95% identity to the native sequences.

15 [0048] The term "sequence identity" as used herein means
that the sequences are compared as follows. The sequences are
aligned using Version 9 of the Genetic Computing Group's GAP
(global alignment program), using the default (BLOSUM62) matrix
(values -4 to +11) with a gap open penalty of -12 (for the
20 first null of a gap) and a gap extension penalty of -4 (per
each additional consecutive null in the gap). After alignment,
percentage identity is calculated by expressing the number of
matches as a percentage of the number of amino acids in the
claimed sequence.

25 [0049] Analogs in accordance with the present invention may
also be determined in accordance with the following procedure.
Polypeptides encoded by any nucleic acid, such as DNA or RNA,
which hybridizes to the complement of the native DNA or RNA
under highly stringent or moderately stringent conditions, as
30 long as that polypeptide maintains the biological activity of
the native sequence are also considered to be within the scope
of the present invention. The native DNA sequence for β2m is
that part of SEQ ID NO:1 that encodes the β2m portion of the
monochain, i.e., nucleotides 1-357 of SEQ ID NO:1. The native

DNA for the portions of HFE in the monochain is that part of
SEQ ID NO:1 which encodes the HFE portion of the monochain,
i.e., nucleotides 403-1227 of SEQ ID NO:1.

[0050] Specificity in hybridization, however, is a function
of the difference in stability between the desired hybrid and
"background" hybrids. Hybrid stability is a function of duplex
length, base composition, ionic strength, mismatching, and
destabilizing agents (if any).

[0051] The Tm of a perfect hybrid may be estimated for
DNA:DNA hybrids using the equation of Meinkoth et al (1984), as

$$Tm = 81.5°C + 16.6 \ (\log M) + 0.41 \ (\%GC) - 0.61 \ (\% \ form) - 500/L$$

and for DNA:RNA hybrids, as

$$Tm = 79.8°C + 18.5 \ (\log M) + 0.58 \ (\%GC) - 11.8 \ (\%GC)^2 - 0.56(\% \ form) - 820/L$$

where

M, molarity of monovalent cations, 0.01-0.4 M NaCl,

%GC, percentage of G and C nucleotides in DNA, 30%-75%,

% form, percentage formamide in hybridization
   solution, and

L, length hybrid in base pairs.

[0052] Tm is reduced by 0.5-1.5°C (an average of 1°C can be
used for ease of calculation) for each 1% mismatching.

[0053] The Tm may also be determined experimentally. As
increasing length of the hybrid (L) in the above equations
increases the Tm and enhances stability, the full-length rat
gene sequence can be used as the probe.

[0054] Filter hybridization is typically carried out at
68°C, and at high ionic strength (e.g., 5 - 6 X SSC), which is
non-stringent, and followed by one or more washes of increasing
stringency, the last one being of the ultimately desired high
stringency. The equations for Tm can be used to estimate the

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appropriate Ti for the final wash, or the Tm of the perfect
duplex can be determined experimentally and Ti then adjusted
accordingly.

[0055] Hybridization conditions should be chosen so as to
5   permit allelic variations, but avoid hybridizing to other
genes. In general, stringent conditions are considered to be a
Ti of 5°C below the Tm of a perfect duplex, and a 1% divergence
corresponds to a 0.5-1.5°C reduction in Tm.

[0056] As used herein, highly stringent conditions are those
10 which are tolerant of up to about 5% sequence divergence.
Without limitation, examples of highly stringent (5-10°C below
the calculated Tm of the hybrid) conditions use a wash solution
of 0.1 X SSC (standard saline citrate) and 0.5% SDS at the
appropriate Ti below the calculated Tm of the hybrid. The
15 ultimate stringency of the conditions is primarily due to the
washing conditions, particularly if the hybridization
conditions used are those which allow less stable hybrids to
form along with stable hybrids. The wash conditions at higher
stringency then remove the less stable hybrids. A common
20 hybridization condition that can be used with the highly
stringent to moderately stringent wash conditions described
above is hybridization in a solution of 6 X SSC (or 6 X SSPE),
5 X Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured,
fragmented salmon sperm DNA at an appropriate incubation
25 temperature Ti.

[0057] The term "active fractions" of the native polypeptide
or analog thereof is intended to cover any fragment thereof
that retains the biological activity, as explained hereinabove
for each of β2m and HFE in the definition of "analog". For
30 example, fragments can be readily generated from the full-
length sequence where successive residues can be removed from
either or both the N-terminus or C-terminus thereof or from
peptides obtained therefrom by enzymatic or chemical cleavage
of the polypeptide. Thus, multiple substitutions are not

involved in screening for active fractions. If the removal of
one or two amino acids from one end or the other does not
affect the biological activity, such truncated polypeptides are
considered to be within the scope of the present invention.
5   Further truncations can then be carried out until it is found
where the removal of another residue destroys the biological
activity.

[0058] "Functional derivatives" as used herein covers
chemical derivatives which may be prepared from the functional
10   groups which occur as side chains on the residues or the N- or
C-terminal groups, by means known in the art, and are included
in the invention as long as they remain pharmaceutically
acceptable, i.e., they do not destroy the biological activity
of the corresponding polypeptide, or the associated monochain,
15   as described herein and do not confer toxic properties on
compositions containing it. Derivatives may have chemical
moieties, such as carbohydrate or phosphate residues, provided
such a fraction has the same biological activity and remains
pharmaceutically acceptable.

20   [0059] Suitable derivatives may include aliphatic esters of
the carboxyl of the carboxyl groups, amides of the carboxyl
groups by reaction with ammonia or with primary or secondary
amines, N-acyl derivatives or free amino groups of the amino
acid residues formed with acyl moieties (e.g., alkanoyl or
25   carbocyclic aroyl groups) or O-acyl derivatives of free
hydroxyl group (e.g., that of seryl or threonyl residues)
formed with acyl moieties. Such derivatives may also include
for example, polyethylene glycol side-chains that may mask
antigenic sites and extend the residence of the complex or the
30   portions thereof in body fluids.

[0060] Non-limiting examples of such derivatives are
described below.

[0061] Cysteinyl residues most commonly are reacted with
alpha-haloacetates (and corresponding amines), such as

chloroacetic acid or chloroacetamide, to give carboxymethyl or
carboxyamidomethyl derivatives. Cysteinyl residues also are
derivatized by reaction with bromotrifluoroacetone, alpha-
bromo- beta-(5-imidazoyl)propionic acid, chloroacetyl
5   phosphate, - alkylmaleimides, 3-nitro-2-pyridyl disulfide,
methyl-2-pyridyl disulfide, p-chloromercuribenzoate, 2-
chloromercuri-4- nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-
diazole.

    [0062] Histidyl residues are derivatized by reaction with
10 diethylprocarbonate at pH 5.5-7.0 because this agent is
relatively specific for the histidyl side chain.
Parabromophenacyl bromide also is useful; the reaction is
preferably performed in 0.1 M sodium cacodylate at pH 6.0.

    [0063] Lysinyl and amino terminal residues are reacted with
15 succinic or other carboxylic acid anhydrides. Derivatization
with these agents has the effect of reversing the charge of the
lysinyl residues. Other suitable reagents for derivatizing
alpha-amino-containing residues include imidoesters such as
methyl picolinimidate; pyridoxal phosphate; pyridoxal;
20 chloroborohydride; trinitrobenzenesulfonic acid; O-
methylisourea; 2, 4-pentanedione; and transaminase-catalyzed
reaction with glyoxylate.

    [0064] Arginyl residues are modified by reaction with one or
several conventional reagents, among them phenylglyoxal, 2,3-
25 butanedione, 1,2-cyclohexanedione, and ninhydrin.
Derivatization of arginine residues requires that the reaction
be performed in alkaline conditions because of the high pK_a of
the guanidine functional group. Furthermore, these reagents
may react with the groups of lysine as well as the arginine
30 epsilon-amino group.

    [0065] The specific modification of tyrosyl residues *per se*
has been studied extensively, with particular interest in
introducing spectral labels into tyrosyl residues by reaction
with aromatic diazonium compounds or tetranitromethane. Most

commonly, N-acetylimidazole and tetranitromethane are used to
form O-acetyl tyrosyl species and 3-nitro derivatives,
respectively.

[0066] Carboxyl side groups (aspartyl or glutamyl) are
selectively modified by reaction with carbodiimides (R'-N-C-N-
R') such as 1-cyclohexyl-3-[2-morpholinyl-(4-
ethyl)]carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethlypentyl)
carbodiimide. Furthermore, aspartyl and glutamyl residues are
converted to asparaginyl and glutaminyl residues by reaction
with ammonium ions.

[0067] Glutaminyl and asparaginyl residues are frequently
deamidated to the corresponding glutamyl and aspartyl residues.
Alternatively, these residues are deamidated under mildly
acidic conditions. Either form of these residues falls within
the scope of this invention.

[0068] The term "derivatives" is intended to include only
those derivatives that do not change one amino acid to another
of the twenty commonly-occurring natural amino acids.

[0069] The term "salts" herein refers to both salts of
carboxyl groups and to acid addition salts of amino groups of
the monochain of the invention or analogs thereof. Salts of a
carboxyl group may be formed by means known in the art and
include inorganic salts, for example, sodium, calcium,
ammonium, ferric or zinc salts, and the like, and salts with
organic bases as those formed, for example, with amines, such
as triethanolamine, arginine or lysine, piperidine, procaine
and the like. Acid addition salts include, for example, salts
with mineral acids, such as, for example, hydrochloric acid or
sulfuric acid, and salts with organic acids, such as, for
example, acetic acid or oxalic acid. Of course, any such salts
must have substantially similar biological activity to the
monochain of the invention.

[0070] In addition, the soluble β2m/HFE monochain can be
tagged for ease of detection and purification. For instance, a

His-tag, which is a stretch of 6 histidines at the C-terminus,
is recognized by commercial antibodies and thus serves as a tag
for immune detection. It provides some other advantages of
affinity purification and concentration of the recombinant
5   protein from supernatants, cell lysates and bacterial lysates.
The affinity purification is based on the specific interaction
of the target protein (through the histidine tag) with an
immobilized ligand (Nickel beads, metal-based chromatography).
The addition of the his-tag to recombinant proteins provides a
10 number of affinity purification strategies that may be applied
to sort out a specific recombinant protein from a mixture, to
capture the desired molecule for interaction studies or to
remove a component from the purified recombinant protein
preparation, such as protease removal in cleavage reactions.
15 For affinity purification strategies, other tags can be used
such as the GST-tag, T7-tag, S-tag, and CBD-tag, which can all
be purified by specific resins, such as those mainly provided
by Novagene.

[0071] The myc-tag, which has the myc-tag epitope Glu-Gln-
20 Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu (residues 418 to 427 of SEQ ID
NO:2), can be used to enable the detection of the recombinant
soluble monochain in cell lysates and/or supernatants in order
to follow up expression and secretion. This tag is recognized
by a well known commercial antibody that allows the detection
25 of the recombinant protein, a detection which is not dependent
on conformation as opposed to antibody 2F5, which is
conformationally dependent and serves only for limited specific
assays.

[0072] A protease cleavage site, such as that for Factor Xa,
30 can be inserted at the N- or C-terminus of the soluble
monochain in order to remove the his-tag or other tags after
the purification steps and before the injection of the soluble
$\beta 2m$/HFE monochain. This step might be required to eliminate a

possible immune response against the tag itself once injected
into the circulation.

[0073] A recombinant construct of the soluble β2m/HFE
monochain according to the present invention can be prepared by
5   recombinantly fusing the native DNA sequence encoding the β2m,
i.e., nucleotides 1-357 of SEQ ID NO:1, or an active fraction
thereof via a DNA sequence encoding the linker portion to a
native DNA sequence encoding for the HFE portion, i.e.,
nucleotides 403-1227 of SEQ ID NO:1, or an active fraction
10   thereof using conventional well-known recombinant DNA
techniques. Alternatively, either or both of β2m and HFE in
the mon0chan may be encoded by a DNA which hybridizes to the
native DNA sequences or can be obtained by hybridization to the
native DNA sequences under highly stringent or moderately
15   stringent conditions. The recombinant construct can be placed
into an expression vector under the control of an appropriate
promoter for expression of the soluble β2m/HFE monochain in a
host cell. The host cell, which is preferably eukaryotic but
may be prokaryotic, when transformed with the expression vector
20   can be used for preparing the soluble β2m/HFE monochain. This
method of preparing the soluble β2m/HFE monochain according to
the present invention involves culturing the transformed host
cell to express and produce the soluble β2m/HFE monochain and
then recovering the produced monochain product.

25   [0074] The recombinant construct encoding the protein
described above, may serve as a platform for efficient drug
delivery into cells and for modulation of iron absorption/
metabolism. Since the TfR is over-expressed in tumors,
metastases, activated lymphocytes operative in autoimmune
30   diseases, and inflammatory disorders, this platform has several
advantages over current therapies. Particularly, it will be
able to modulate iron metabolism and, consequently, arrest cell
proliferation and activation. In addition, since the monochain
is soluble, but as stable as the natural HFE molecule, it could

be administered for treatment of iron overload disorders and
the like.

[0075] To use the monochain as a platform for drug delivery,
a therapeutic may be bound to the monochain. The bond may be,
5   for example, a covalent bond or a peptide bond. In each
instance, however, a simple assay may be conducted to determine
that the assembled monochain-therapeutic retains the ability of
the monochain to bind TfR. Preferably, the therapeutic is a
protein or polypeptide which is fused to the monochain via a
10 peptide bond. In a preferred embodiment, it is fused to the C-
terminus of the monochain. The experiments herein establish
that an additional protein, which is green fluorescent protein
(GFP) in the examples, can be fused to the C-terminus of the
monochain without affecting the ability of the monochain to
15 bind TfR. The therapeutic may also be fused to the N-terminus
as long as it is experimentally established that it does not so
affect the conformation of the monochain that it no longer
binds TfR.

[0076] When the monochain-therapeutic is a fusion protein,
20 it is preferably made by designing an appropriate DNA construct
which encodes the fusion protein. The monochain-therapeutic in
which the therapeutic is a protein or polypeptide fused to the
soluble β2m/HFE monochain by a peptide bond can either be
constructed by fusing the DNA encoding the therapeutic protein
25 or polypeptide to the recombinant DNA construct of the soluble
β2m/HFE monochain or be constructed by fusing the therapeutic
protein or polypeptide after the soluble β2m/HFE monochain has
been prepared using conventional peptide chemistry techniques.
When the recombinant DNA construct of the soluble β2m/HFE
30 monochain is fused to the DNA encoding the therapeutic protein
or polypeptide to the monochain to generate a further
construct, this further construct can be placed into an
expression vector under the control of an appropriate promoter
for expression of the monochain-therapeutic in a host cell.

The host cell, which is preferably eukaryotic but may be
prokaryotic, when transformed with the expression vector can be
used for preparing the monochain-therapeutic. This method of
preparing the monochain-therapeutic according to the present
5   invention involves culturing the transformed host cell to
express and produce the monochain-therapeutic and then
recovering the produced monochain-therapeutic product.

[0077] A target for proteolytic cleavage by intracellular
proteases, preferably in the endosomes and in the cytosol, can
10 be introduced, i.e., recombinantly in the above monochain-
therapeutic, between the therapeutic drug and the soluble
$\beta2m/HFE$ monochain in order for the transported therapeutic drug
to be cleaved immediately after it penetrates the cell and to
exert its function. Most transported substances require
15 interaction with their specific target at specific locations
inside the cell and, hence, need to be released from the
monochain carrier.

[0078] The genetically engineered monochain of the present
invention can be utilized as a platform for advanced delivery
20 of drugs and other chemicals into cells, and for the regulation
of iron metabolism in target cells. This protein encompasses
two partners:

        1.   The $\beta2m$ molecule that confers stability and
              proper conformation required for trafficking and
25   function in iron regulation.
        2.   A polypeptide derived from the HFE protein that
              is essential for TfR binding and for the
              regulation of iron metabolism in target cells.

[0079] The necessity for such a system is apparent,
30 especially when violent cancer cells become drug resistant and
escape to the central nervous system, which is inaccessible to
most chemotherapeutics. Drug delivery to the brain and down-
regulation of iron consumption of metastasizing cells is
expected to be a solution to these specific problems.

Moreover, in cases where autoimmune cells are activated or in graft implantations, biotechnological tools that down-modulate such cells are required. The present data show that most tumor cell lines, as well as activated lymphocytes express

5   constitutively high levels of TfR, the receptor that is targeted by β2m/HFE monochain.

[0080] Three forms of the recombinant protein were engineered for different purposes. Two forms harbor a His-tag at the N-terminus (β2m/HFE/Mh) and at the C-terminus

10 (β2m/HFE/Mhm) of the molecule. The third form harbors a Green Fluorescent Protein (GFP) (Zhang et al, 1996) protein that is fused to the C-terminus of the monochain (β2m/HFE/Mg). The different forms of the monochain are expressed in HEK293 cells and are secreted to the surrounding media. Both the β2m/HFE/Mh

15 and β2m/HFE/Mhm molecules bind to TfR with similar affinities; however, only the β2m/HFE/Mhm protein is properly folded, as verified by its recognition with conformation-dependent antibody, 2F5. The fact that the binding of β2m/HFE to TfR is independent of proper conformation will allow the conjugation

20 of peptides or polypeptides to the N-terminus of the monochain despite their potential to affect β2m/HFE conformation. Such molecules could deliver drugs but might not have a function in iron metabolism. The β2m/HFE/Mhm and β2m/HFE/Mg molecules are soluble and stable in a serum-rich medium. Therefore,

25 apparently, they are resistant to serum proteases, an advantageous property for a drug delivery vehicle that should be administered into the circulation. When synthesized in cells or added to human cell lines in the culture media, both β2m/HFE/Mhm and β2m/HFE/Mg molecules penetrate target cells

30 rapidly, within 1 hour. When β2m/HFE monochain is chemically linked to another molecule (FITC conjugated molecule) or synthesized as an engineered protein (GFP monochain), it is able to deliver chemicals or proteins into cells. β2m/HFE monochain administered in the culture media, penetrates

efficiently into human cells in a transferrin-dependent manner
and co-localizes, at least partially, with transferrin
containing vesicles. Therefore, the monochain and its
derivatives specifically target TfR on the surface of human
5   tumors, and could reach the endosomes, the desired
intracellular target organelle. The present data also indicate
that the β2m/HFE/Mg reaches other intracellular organelles.
Interestingly, the results imply that the human TfR requires
co-binding of its natural ligand, the human transferrin, in
10   order to promote efficient binding of the monochain to TfR or
efficient internalization of monochain-TfR complexes. Since
transferrin is present in large excess in the circulation, it
is not a limiting factor for the binding of β2m/HFE monochain
to the TfR.

15   [0081] The β2m/HFE monochain is able to target and deliver
drugs into human TfR-expressing cells. Since transferrin-based
drug delivery was shown to overcome drug resistance of tumor
cells and also to penetrate the brain, it is expected that
β2m/HFE monochain-based therapy will operate in a similar
20   manner.

     [0082] Peptide and protein therapeutics are generally
excluded from transport from blood to brain, owing to the
negligible permeability of these drugs to the brain capillary
endothelial wall, which makes up the blood-brain barrier *in*
25   *vivo*. However, peptides or protein therapeutics may be
delivered to the brain when a non-transportable therapeutics is
coupled to a BBB drug transport vector. The principle of the
drug delivery into the brain tissue is when the transport
vector binds to the luminal plasma membrane of endothelial
30   cells via the interaction of the transport vector with a cell
surface receptor. The binding step then initiates transcytosis
of the whole complex across the endothelial cell and results in
the release of the transported vector into the brain
extracellular fluid. When a cleavable linker between the

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transport vector and the drug, such as disulfide bond is
employed, tissue enzymes may cleave the drug from the vector.

[0083] Receptor-mediated transcytosis across the BBB has to
date only been shown for insulin and transferrin where the
latter is necessary for the cellular delivery of iron into the
brain. Binding of transferrin to the TfR at the cell surface
membrane, leads to internalization of the transferrin in
vesicles, that are further headed for exocytosis at the luminal
plasma membrane, via vesicular trafficking through
intracellular compartments. Due to its high endogenous plasma
concentration of 25 uM, transferrin itself may be limited as a
drug transporting vector, since the transferrin receptors are
almost saturated under physiologic conditions. As an
alternative approach, anti-TfR antibody was demonstrated to be
an efficient transport vector, although it presents problems of
immune response. A different ligand of the TfR, the soluble
β2m/HFE monochain, that is not affected by the transferrin-
saturation condition of the receptor and undergoes
internalization as the transferrin does, would serve as an
optimal BBB vector.

[0084] Since the β2m/HFE monochain was engineered from two
polypeptides operating in concert to down-modulate iron uptake,
opposite to the effect of transferrin, this molecule is
advantageous over the transferrin molecule. The main outcome
of treatment with β2m/HFE monochain is the decreased level of
TfR in treated cells. This result is compatible with another
result: following 2 hours of treatment of cells with
β2m/HFE/Mg there was hardly any detectable level of β2m/HFE
monochain residing in cells. Since at 2 hours of incubation
the monochain was hardly detected in cells, although it was
present in large excess in the conditioned medium, it is highly
feasible that the complex monochain/TfR was directed towards
degradation. This process would result in decreased level of
functional TfR on the cell surface. It appears that following

the binding to its cognate receptor, the β2m/HFE monochain
leads the TfR to degradation, thereby preventing further TfR-
dependent iron uptake.

[0085] The conclusion that β2m/HFE monochain affects iron
metabolism is clearly supported by the results of the attempt
to over-express HFE and β2m/HFE monochain in tumor cell lines.
It was observed that over-expression of HFE as well as β2m/HFE
in HeLa and PC3 human tumor cell lines confers a yet
unidentified stress resulting in the rapid degradation or
blocking of synthesis of HFE and β2m/HFE. Since TfR is the
main iron uptake system in dividing cells, it is expected that
the expression of HFE-derived molecules could inhibit iron
uptake and, consequently, cause growth arrest and death. These
data strongly corroborate the conception that the monochain can
be used as a biotechnological tool for causing growth arrest of
highly proliferating cells.

[0086] While the examples of the present invention relate
mostly to cancer therapy and treatment of autoimmunity, the
monochain could be used in any condition that would benefit
from such a treatment.

EXAMPLE 1:   General Techniques

Cell Culture

[0087] Cells were maintained in Dulbecco's Modified Eagle's
Medium (DMEM, GibcoBRL, Life Technologies Ltd, Palsley,
Scotland), supplemented with 2 mM glutamine (Biological
Industries, Beit Haemek, Israel), 10% fetal calf serum (FCS,
HyClone Laboratories, Inc, Logan, Ut, USA), penicillin,
streptomycin, gentamycin and amphotericin B (Biological
Industries, Beit Haemek, Israel). For certain experiments, as
well as for purification of the monochain molecule from culture
medium, a low protein synthetic medium DCCM-1 (Biological
Industries, Beit Haemek, Israel) was used.

Cell Lines

[0088] The following cell lines were used: human embryonal
kidney HEK293 (ATCC), mouse embryonal fibroblasts transformed
with adenovirus 12, VAD12.79, mouse melanoma, B16, HeLa (ATCC),
5 human neuroblastoma cell lines, SK-NSH, Nub7, NHB, WN, human
hepatoma cell lines Hep-G2, SK-HEP1, HEP3bp5, HuH7, human
cervical squamous carcinoma, SiHa, human cervical epidermal
carcinoma, Caski, human melanoma cell lines GTBS, WUBI, FO1,
human ovarian carcinoma cell lines HEY, HEYC2, OVCAR, OC109,
10 human breast carcinoma cell lines T47D, MDA 231, human squamous
carcinoma cell lines 22A, 14C-16, human Burkitt's lymphoma cell
lines, Daudi, DG-75, human pre B, Nalm6, human T leukemia,
Jurkat, human chronic myelogenous Leukemia cell lines K562,
Dami, Meg01, human promyelocytic leukemia HL-60.
15 Antibodies

[0089] The following monoclonal antibodies (mAbs) were used;
R17 217.1.3 (Lesley et al 1984) (anti-mouse TfR, ATCC TIB-219),
S19 (Tada et al, 1980) (anti-mouse β2m, a kind gift of Dr. T.
Hansen, Washington University, St Louis), BBM1 (anti-hβ2m, ATCC
20 HB-28), anti-HFE (CT) (rabbit anti-hHFE antisera prepared by
immunization of rabbits with peptide (C)RKRQGSRGAMGHYVLAERE
(SEQ ID NO:3) that corresponds to the sequence of the
cytoplasmic tail of hHFE), B2.62.2, anti hβ2m, kindly provided
by F. Lemonnier, Unité d'Immunité Cellulaire Antivirale,
25 Insititut Pasteur, Paris, France, V1-10 (anti-human TfR, a kind
gift of Dr. Z. Eshhar, The Weizmann Institute of Science,
Rehovot, Israel), H68.4, anti-TfR (mouse and human) Zymed
Laboratories, San Francisco, CA), anti MAP kinase polyclonal
antibodies (442704-S) (Sigma), anti myc tag (9E10 hybridoma)
30 (Fan et al, 1998).

Northern Blot Analysis and RT-PCR

[0090] RNA extraction and Northern blot analysis were
performed as previously described (Rotem-Yehudar et al, 1994,
Fromm et al, 1998). Reverse transcription of RNA was carried

out for 1 hour at 42°C, in 20 μl PCR buffer containing 1 μg of
total cellular RNA, 0.5 μg Oligo d(T)18 primer (Promega,
Madison, WI, USA), nucleotide mixture (1 mM), 24 units
ribonuclease inhibitor (MBI FermentasVilnius, Lithuania), and
5   15 units AMV reverse transcriptase (Promega). PCR was carried
out in the appropriate buffer with 1 μl of the cDNA, 25-50
pmole of the relevant primers, 40 μM nucleotide mixture, and
1.54 units Taq DNA polymerase (Biological Industries). After
the appropriate number of PCR cycles (MiniCycler, MJ Research
10 Inc. Watertown, MA, USA), 10 μl or less of the reaction mix
were fractionated on a 1% agarose gel. Specific conditions are
described in the section "Specific Techniques".

Primers

    Actin- *Forward* 5' GTTTGAGACCTTCAACACCCC 3' (SEQ ID NO:4)
15   *Reverse* 5' GTGGCCATCTCTTGCTCGAAGTC 3' (SEQ ID NO:5)
    HFE- *Forward* 5' GGGGCGCTTGCTGCGTTCACAC 3' (SEQ ID NO:6)
        Reverse 5' GGTCCAAAACACCTCTCCCCAGCTCC 3' (SEQ ID
              NO:7)

[0091] Other specific primers are described in the "Specific
20 Techniques" section.

FACS® Analysis

[0092] Cells were harvested by mild trypsinization, followed
by washes in media supplemented with 5% FCS and 0.01% sodium
azide. About 10^6 cells were incubated at 4°C with the
25 appropriate concentration of the first antibody for 45 minutes,
washed and then incubated in the dark for another 30 minutes
with the second antibody. The cells were then washed with PBS
and fluorescence intensity was analyzed with a Becton-Dickinson
Cell Sorter (Becton-Dickinson & Co., Mountain View, CA, USA).
30 Metabolic Labeling, Immunoprecipitation and Western Blot
Analysis

[0093] Cells were grown to 80% confluence and starved for 60
minutes in methionine-free medium. They were then labeled in
methionine-free medium containing 150 μCi/ml ^{35}[S]-methionine

(Amersham Pharmacia Biotech, Little Chalfont, UK) for 30
minutes, washed with PBS and chased for the indicated
intervals. The cells were lysed with buffer containing 0.5%
NP-40, 50 mM Tris (pH 7.5), and 150 mM NaCl, immunoprecipitated

5   with the indicated antibodies and Protein A (Boehringer
Mannheim GmbH). The immunoprecipitates were washed with buffer
containing 0.1% NP-40, 50 mM Tris (pH 7.5), and 150 mM NaCl.
For endo H-treatment, immunoprecipitates were eluted by adding
20 µl buffer containing 100 mM Tris (pH 8), 1% SDS and boiling

10 for 5 minutes. The samples were centrifuged and the
supernatant was added to 2 µl of 50 mM citrate buffer (pH 5.5)
containing 1000 units of endo H (New England Biolabs, Beverly,
MA, USA). The samples were incubated at 37°C for 18 hours,
followed by the addition of sample buffer. All the buffers

15 contained the following protease inhibitors: 1 mM PMSF, 10
µg/ml aprotinin, and 10 µg/ml leupeptin (Sigma Chemical Co.).
The protein concentration was determined with a Bicinchronic
Acid Protein Assay Kit (Sigma Chemical Co.) and equivalent
amounts of protein were loaded on the gels. Following

20 fractionation of the immunoprecipitates on 12-15% SDS-PAGE, X-
OMAT AR X-ray films (Eastman Kodak Company, Rochester, NY, USA)
were exposed to the dried gels.

[0094] For Western blot analysis, the immunoprecipitates or
cell extracts were transferred to nitrocellulose (Amersham

25 Pharmacia Biotech). The membrane was hybridized with the
specific antibody, followed by the appropriate horseradish
peroxidase-conjugated secondary antibodies and
chemiluminescence was measured (Amersham Pharmacia Biotech)
according to the manufacturer's directions.

30 Quantitation of Radioactive Signals

[0095] Dried radioactive gels or blots were exposed to a
phosphoimager screen and analyzed with a phosphoimager (Fuji
BAS1000, Tokyo, Japan).

Immunocytochemistry

[0096] Sub-confluent cells grown on coverslips (11 mm:Menzel glass, Merk, Germany) were incubated with either 20 μg/ml of β2m/HFE monochain or 5 μg/ml of tetramethylrhodamine-conjugated transferrin (Molecular Probes). The cells were washed twice with PBS (pH 7.4) and fixed in methanol for 5 minutes at 4°C and in methanol/acetone (1:1) for 5 minutes at 4°C. The cells were washed 3 times for 5 minutes in PBS. The coverslips were incubated for 60 minutes with anti-human β2m antibodies (B2.62.2) followed by 3 washes of 5 minutes with PBS. The coverslips were incubated for 60 minutes with Cy2-conjugated goat anti mouse antibodies or Cy3-conjugated goat anti mouse antibodies (Jackson laboratories), washed 4 times for 5 minutes with PBS, mounted with Gel-Mount (Biomeda Corp.) and then analyzed by immunofluorescence microscopy and Confocal microscopy.

EXAMPLE 2: Specific Techniques and Generation of Tools for Physical, Biochemical and Functional Analysis of the β2m/HFE Monochain

Production of Anti-hHFE Monoclonal Antibodies

[0097] The hHFE-transfected clone of VAD12.79 that expresses the human HFE (WH6) (generated in Dr. Ehrlich laboratory) was used for syngeneic immunization of C57Bl/6 mice. The mice received one subcutaneous injection of 10^6 cells in CFA and then five additional injections (one injection every 4 weeks) of 10^6 cells each. One day prior to removal of the spleen, the mice were immunized intravenously with 10^5 irradiated cells. Hybridization of the spleen cells with NSO myeloma cells, subsequent culture, and cloning were performed as described (Kohler et al, 1975). Monoclonal antibodies (mAbs) were selected according to their specific binding to WH6 cells as analyzed by FACS. The mAb 2F5, directed against a

conformation-dependent epitope on HFE/β2m heterodimer was
utilized for further immunoprecipitation and FACS analyses.

Construction of Plasmids Encoding Soluble β2m/HFE Monochain

[0098] A single-chain HFE gene expression vector in which a
5 full-length β2m gene linked via its 3' end (the region encoding
the carboxy-terminus of the molecule) to the 5' end (the region
encoding the amino-terminus of the α1-α3 domains) of the human
HFE gene through an oligonucleotide spacer (encoding the 15-
amino acid peptide (GGGGS)₃ (SEQ ID NO:8)), was constructed.
10 The construct was obtained by splicing overlap extension PCR.
The chimeric PCR product was introduced into BamHI-XhoI sites
of the mammalian expression vector pcDNA3.1/his C (Invitrogene)
and into BamHI-XhoI sites of the mammalian expression vector
pcDNAHis/Myc A (Invitrogene). The β2m was amplified from a
15 plasmid harboring human β2m gene, with a 5' primer (P1) that
incorporates a BglII restriction site followed by the 5' end of
the β2m leader sequence, and a 3' primer (P2) that incorporates
the 3' terminus of the β2m coding region and 39 bases encoding
the 5' region of the peptide spacer.

20   P1: 5' ggcacga<u>agatct</u>gagatgtctcgctccgtgg 3' (SEQ ID NO:9)
              (The BglII site is underlined.)

         P2: 5' gccgccacc<u>ggatcc</u>acctccgcccgagccgccacctcc<u>catgtctcga</u>
              <u>tcccacttaac</u> 3' (SEQ ID NO:10) (The BamHI site and β2m
              terminus are underlined respectively.)

25 [0099] The human HFE cDNA was isolated from human liver cDNA
library. Following amplification with the relevant primers,
the 5' primer, P3, and the 3' primer, P4. P3 encodes 18-bp
overlap with the 3' end of P2, 6 bases encoding the 3' region
of the spacer, and the 5' region of the HFE α1 domain. P4
30 encodes the 3' end of the HFE α3 domain and an XhoI restriction
site.

P3: 5′ ggctcgggcggaggt<u>ggatcc</u>ggtggcggcggttcc<u>cgcttgctgcg</u>
     <u>ttcacac</u> 3′ (SEQ ID NO:11;) (The BamHI restriction
     site and the α1 domain are underlined, respectively.)

P4: 5′ gccagacgg<u>ctcgag</u>ctcccagatcacaatgagggg 3′ (SEQ ID
     NO:12) (The XhoI site is underlined)

[0100] The P3/P4 HFE amplification product was mixed in a
splicing overlap extension PCR with the P1/P2 β2m gene product
following by amplification with the 5′ primer P1 and the 3′
primer P4. This amplification resulted in a single construct
incorporating the leader – β2m – spacer – HFE (α1-α3) coding
sequences flanked by 5′ BamHI and 3′ XhoI restriction sites.
P1/P2, P3/P4, PCR were carried out as follows: 30 cycles of
95°C for 90 seconds, 60°C for 1 minute, and 72°C for 2 minutes.
The final overlapping PCR was performed by a single cycle of
95°C for 90 seconds, 50°C for 1 minute, and 71°C for 150
seconds without primers to facilitate annealing of the β2m and
HFE fragments, following by 45 cycles of 95°C for 90 seconds,
60°C for 1 minute, and 72 °C for 160 seconds in the presence of
primers P1 and P4. The final PCR product was enzymatically
cleaved by BglII-XhoI sites and was ligated in frame into
pcDNA3.1/His and pcDNAHis/Myc (Invitrogene) using the
BamHI/BglII-XhoI cloning sites. The correct sequence of the
full-length insert was verified by sequencing using relevant
primers. β2m/HFE monochain in pcDNA3.1/His is designated
β2m/HFE/Mh. β2m/HFE monochain in pcDNAHis/Myc is designated
β2m/HFE/Mhm.

Construction of a Three Partner-Monochain Encoding a
GFP/Monochain Chimera

[0101] A monochain of human soluble β2m-HFE-EGFP(His/Myc)
was constructed by cloning in frame cDNA encoding EGFP (Zhang
et al, 1996) into the β2m/HFE/Mhm expression vector. The EGFP
was cloned downstream of the β2m/HFE monochain sequence but
upstream of the His/Myc epitope sequences. EGFP cDNA was

amplified by PCR from pEGFP.C2 vector (Clontech Laboratories
Palo Alto, CA, USA) using the primers:

    EGFP   *Forward* – 5' CCAT<u>CTCGAG</u>GTCGCCACC**ATGGTGAG** 3' (SEQ ID
            NO:13)

    EGFP   *Reverse* – 5' GC<u>TCTAGA</u>GG**ACTTGTACAGCTCGTCC** 3' (SEQ ID
            NO:14)

[0102] The underlined sequences indicate restriction digest
sites while bold sequences demonstrate EGFP coding sequences.

[0103] The EGFP Forward primer incorporated XhoI restriction
site and leader sequence from EGFP, while EGFP Reverse primer
included XbaI restriction site and sequence encoding for the
carboxy terminus of EGFP excluding STOP codon. EGFP PCR
product was amplified in a standard PCR reaction buffer with
2.5 U of Taq polymerase (Takara Shuzo, Shinga, Japan) at 30
cycles of the following PCR condition: denaturation at 94°C
for 30 seconds; annealing at 55°C for 30 seconds and; extension
at 72°C for 30 seconds. The PCR product was digested with XhoI
and XbaI and cloned in frame into the corresponding sites in
the β2m/sHFE/Mhm vector. The resulting β2m/HFE/GFP vector was
analyzed by a diagnostic restriction digest and correct
sequence confirmed by automated sequencing. β2m/HFE monochain
in pGFP is designated β2m/HFE/Mg.

Stable Transfection

[0104] HEK293, HeLa and VAD12.79, were transfected by
calcium phosphate DNA co-precipitation as described (Fromm et
al, 1998). The plasmid DNA contained either 10 µg of the
hHFEcDNA (human HFE), mutant hHFEcDNA (C282Y), β2m/HFE/Mhm,
β2m/HFE/Mh and β2m/HFE/Mg or human TfR cDNA. Individual clones
were selected and expanded as described previously (Fromm et
al, 1998). The stably transfected cells are designated as
follows: 293/mutHFE, 293/HFE, 293/β2m/HFE/Mh, 293/β2m/HFE/Mhm,
293/β2m/HFE/Mg, HeLa/β2m/HFE/Mhm, HeLa/mutHFE, HeLa/HFE, and
VAD12.79/hTfR.

Purification of Secreted β2m/HFE Monochain Protein from
Conditioned Media

[0105] 300-500 ml of DCCM-1 supernatants collected from
293/β2m/HFE/Mhm-secreting cells, were incubated with 10 ml of
5   His-bind beads (Novagene) at 4°C for 2 hours, followed by
washing with 300 ml of phosphate-buffered saline (PBS) at pH
7.4. Elution was carried out using 5 ml of 200 mM of
Immidazole in PBS at pH 7. Eluates were tested for protein
concentration by bicinchoninic acid (BCA) assay (Pierce),
10   dialyzed against PBS and fractionated on SDS-PAGE.

Chemical Linkage of FITC to Purified β2m/HFE Monochain

[0106] Isothiocyanate labeling procedure was carried out as
described in Antibodies: A Laboratory Manual (Harlow and Lane,
1998 (Chapter 9, pp. 354-355). 200 µg of purified protein was
15   adjusted to pH 9 in 0.1 M sodium carbonate. Fluorescein
isothiocyanate (FITC) (Sigma) was freshly dissolved in 1 mg/ml
dimethyl sulfoxide and 10 µl of FITC solution was added to the
protein. The reaction was carried out in the dark for 8 hours
at 4°C. NH_4Cl was added to a final concentration of 50 mM and
20   unbound FITC was separated from the conjugate by dialysis
against PBS for 48 hours at 4°C.

RESULTS

Expression of HFE and TfR in Human Tumor Cell Lines and in
Human Activated Lymphocytes

25   [0107] 30 human tumor cell lines of different origin have
been screened for the expression of HFE and TfR genes by
Northern blot analysis (Figs. 1a-1c). The data demonstrate
that, except for neuroblastomas, all the tumors express TfR.
Tumors of lymphoid origin, melanomas, cervical carcinomas,
30   breast cancer and some hepatomas express very high TfR levels.
The expression of HFE is variable and does not directly
correlate with TfR expression. The TfR signal was evident
following 4h exposure of the labeled blot to X-ray film, while

the HFE signal was detected following 2 weeks exposure of the
same blot to X-ray film. Thus, it is apparent that HFE
expression in human tumors is relatively low while the TfR
expression is remarkably high. These results imply that the
5 TfR is the main iron uptake system in tumors, and HFE is its
adversary negative regulator.

[0108] Cell surface expression of TfR was analyzed by FACS
(Figs. 1d-1k). The data show that the receptor is widespread
and highly expressed on the surface of various human tumors.
10 Thus, any of the human tumor cell lines can be used as a target
for testing therapeutic utilities that are based on inhibition
of iron uptake by TfR. The expression of HFE and TfR genes in
resting versus activated human lymphocytes was tested (data not
shown). These assays demonstrated that the expression of TfR
15 is induced dramatically following activation, as reported
previously (Bayer, 1999, Drobyski, 1996). Hence, lymphocyte
activation depends on iron supply and TfR expression.
Consequently, interference with iron uptake may lead to
lymphocyte inactivation. This system may serve as a model for
20 inactivation of autoimmune lymphocytes.

Sequence Analysis of Plasmid Encoding a Soluble β2m/HFE
Monochain

[0109] Using recombinant DNA techniques a recombinant DNA
fragment was engineered containing β2m and the α1-α3 region of
25 human HFE that are linked via a DNA linker (described in
"Specific Techniques"). The individual fragments were
amplified by PCR and cloned into expression vectors harboring
his-tag. DNA and amino acid sequences are presented (Fig. 2).

HHFE-Transfected Cells and β2m/HFE Monochain Transfected Cells
30 Express and Secret the Encoded Molecules, Respectively

[0110] Human embryonal kidney cells transfected with HFE and
derivatives (HFE, mutHFE, β2m/HFE/Mh and β2m/HFE/Mhm) were
selected and screened for the expression of the transfected
gene by RT-PCR using the relevant primers. Positive clones

expressing the transfected genes were chosen for further
analysis. 293/HFE and 293/mutHFE clones express the encoded
protein as determined by Western blot analysis of lysates from
these clones, using antibodies directed against HFE-cytoplasmic
5   tail (HFE-CT, data not shown). Cell extracts and conditioned
media from clones positive for β2m/HFE/Mhm were tested by
Western blot analysis for the expression of the encoded protein
using anti-myc antibodies (9E10 hybridoma) (Fig. 3). The
results show that the β2m/HFE/Mhm protein is expressed and is
10 being secreted to the medium. The monochain is stable in 10%
serum for at least 24 hours.

The β2m/HFE Monochain Binds to TfR

[0111] The association of human HFE and β2m/HFE monochain
with TfR was tested. Following metabolic labeling and
15 immunoprecipitation of representative clones with anti-TfR (V1-
10) and anti-hβ2m (B2.62.2) monoclonal antibodies, the
expression of β2m/HFE monochain or HFE that are not associated
with TfR was detected with anti-HFE antibody, 2F5. The latter
recognize an epitope on β2m/HFE complexes which is masked
20 following binding to TfR (Vahdati et al, submitted to
publication). Fig. 4 summarizes the data for HEK293 cells,
293/HFE, 293/β2m/HFE/Mh and 293/β2m/HFE/Mhm. The data
demonstrate that the parental 293 cells express TfR, β2m (12
kD, mostly associated with class I heavy chains (45 kD)), and
25 do not express any detectable level of endogenous HFE. 293/HFE
cells express HFE/β2m heterodimers (as detected by 2F5) and
TfR-associated HFE/β2m complexes, as detected mainly by anti-
β2m (B2.62.2) and to much lower extent by anti-TfR (V1-10).
293/β2m/HFE/Mh cells express the expected monochain protein (55
30 kD) as indicated by its co-precipitation with anti-TfR (V1-10),
but both HFE and β2m epitopes were not recognized by the
respective mAbs, suggesting that the recombinant protein is not
properly folded. 293/β2m/HFE/Mhm cells express the expected
protein (55 kD). The protein associated with TfR as indicated

by its co-immunoprecipitation with anti-TfR mAb (V1-10), and is
properly folded since it was recognized by anti-HFE (2F5) and
anti-β2m (B2.62.2) mAbs. The fact that the association of the
β2m/HFE/Mh protein was similar to that of the β2m/HFE/Mhm,
implies that binding of HFE proteins to the TfR requires a
linear sequence element that is independent of conformation.
The following conclusions can be drawn from these data:

        A.   Proper folding of β2m/HFE monochains is
            influenced by the location of the His-tag.

        B.   In cells, β2m/HFE monochains are able to bind
            TfR independent of their conformation and as
            efficient as the wild type w.t. HFE.

Synthesis and Intracellular Trafficking of the β2m/HFE Monochain, HFE and TfR Molecules

[0112] HEK293, HEK293/HFE and HEK293/β2m/HFE/Mhm cells were
metabolically labeled for 30 min, chased, lysed in NP40
containing buffer and immunoprecipitated with the relevant
mAbs. Immunoprecipitates were fractionated on SDS-PAGE (Figs.
5a and 5b). The data in Fig. 5a show that the intracellular
half-life of the β2m/HFE/Mhm molecule is ~8 hours. The β2m/HFE
monochains co-immunoprecipitate with TfR (see co-
immunoprecipitation with anti-TfR mAbs, V1-10). However, the
half-life of the TfR-associated molecules is ~2 hours,
indicating that these complexes dissociate readily. In the
supernatant β2m/HFE/Mhm is evident already at 1 hour, and it
appears to be stable and accumulates during the following 24
hours.

[0113] Fig. 5b shows that TfR-associated β2m/HFE complexes
have a longer half-life (more than 24 hours).

[0114] The observation that the HFE signal in the TfR
associated complexes increased during the first 4 hour chase,
while the TfR signal decreased, suggesting that newly
synthesized TfR associates first with "cold" HFE and, at a

later time, points surface TfR binds labeled β2m/HFE complexes
at the cell surface.

[0115] The compiled data demonstrate that:

A.   Most of the β2m/HFE/Mhm molecules are
      transported rapidly through the secretory
      pathway and are secreted to the media. Some of
      these molecules associate with TfR but have a
      relatively short half-life.

B.   β2m/HFE complexes might have the capacity to
      associate with TfR on the cell surface.

C.   β2m/HFE/Mhm molecules are stable in the
      supernatant for at least 24 hours.

HFE and β2m/HFE Monochain Cannot Be Over-Expressed in Some
Human Tumor Cell Lines

[0116] The ability of HFE to inhibit iron uptake and reduce
intracellular iron pools raised the possibility that over-
expression of HFE may cause growth arrest of tumor cells that
utilize the TfR as the main iron uptake system. To test this
possibility directly and to determine whether the β2m/HFE
monochain can exert the same effect, HeLa cells were
transfected with wild type HFE, mutC282Y/HFE and β2m/HFE/Mhm
expression vectors.

[0117] G418 resistant clones were selected and screened by
RT-PCR and immunoprecipitations for the expression of the
transfected genes. The total number of individual G418
resistant clones in each transfection was the same. The
results of RT-PCR on mixed populations from each transfection
are shown in Fig. 6a. The data demonstrate that mixed
population from each transfection express comparable levels of
the transfected gene. However, when tested for protein
expression by immunoprecipitation analysis with the relevant
antibodies only the monochain, but not the wild type HFE, could
be detected (Fig. 6b, immunoprecipitation with anti-TfR, V1-10
and with anti HFE, 2F5). Moreover, analysis of individual

clones revealed that in monochain transfected clones that are
G418 resistant, only 30% express the β2m/HFE monochain as
determined by immunoprecipitation, data not shown. Therefore,
the expression of HFE and β2m/HFE monochain appears to be down-
5 regulated at the post-transcriptional level in HeLa cells. A
similar phenomenon was observed in a prostate cancer cell line
(PC3) transfected with the HFE gene, data not shown. It
appears that the wild-type HFE, and to a lesser extent the
β2m/HFE monochain, are subjected to negative regulation in
10 certain human tumor cells (in HeLa and PC3 but not in HEK293
cells). The data suggest that the expression of the HFE and
HFE derivatives interferes with cell growth and/or survival.
Radiolabeled β2m/HFE Monochain Binds to TfR on the Surface of
Target Cells
15   [0118] HEK293/β2m/HFE/Mhm cells were metabolically labeled
for 4 hours, washed and incubated in DCCM-1 for overnight. The
media enriched in radiolabeled monochain was collected and
further incubated with mouse and human cultured tumor cell
lines. Binding to TfR was examined following
20 immunoprecipitation of TfR-associated HFE complexes from cell
lysates at 1 and 24 hours (Figs. 7a and 7b). Fig. 7a shows
that the β2m/HFE monochain co-immunoprecipitated with TfR
expressed by target cells. In the human cell lines (FO1 and
HeLa), β2m/HFE monochain signal was observed following 1 hour
25 incubation with target cells and was increased at 24 hours. In
mouse cells (VAD12.79, B16) a signal was observed following 1
hour of incubation, but no signal was evident at 24 hours.
Interestingly, in VAD12.79/hTfR cells, accumulation of β2m/HFE
complexes could be detected at 24 hours. The binding of human
30 HFE to mouse TfR is of very low affinity (Vahdati et al,
submitted) supporting the fact that such complexes may
penetrate cells inefficiently. Identical data are shown in
Fig. 7b. This figure also demonstrates that β2m/HFE complexes
(not associated with TfR, as indicated by their

immunoprecipitation with 2F5) accumulated in human cells. The
latter phenomenon could result from dissociation of TfR-
associated complexes inside the cells. The different
accumulation pattern of β2m/HFE monochain in mouse and human
5   cells suggests that the trafficking pathway of the β2m/HFE
monochain in cells is dictated by its binding to human TfR.
The data in these experiments substantiate the ability of the
monochain to target cells specifically via the TfR, and to
accumulate in cells for a relatively long period of time.

10 Purified β2m/HFE Monochain Penetrates the Cells and Co-
Localizes with Transferrin
      [0119] β2m/HFE monochain was purified from HEK293/β2m/HFE/
Mhm conditioned media as described in the "Specific Techniques"
section. The 55 kD monochain was clearly detected by Coomassie
15 blue staining (Fig. 8, upper panel) and Western blot analysis
using anti-myc antibodies (Fig. 8, lower panel). The purified
β2m/HFE was tested for its potential to penetrate cells.
VAD12.79 cells grown on cover glass slides were incubated with
the monochain, rhodamine-labeled-transferrin or both. The
20 cells were fixed and stained with anti-human β2m antibodies and
a fluorescent-labeled secondary relevant antibody. Analysis
was performed by confocal microscopy (results not shown). The
results show that the monochain is localized to vesicular
punctuated structures in the cytoplasm of the cells, suggesting
25 that it was internalized into endocytic vesicles, like
transferrin (Fig. 9a). Moreover, it is evident that at least
part of the monochains is co-localized to the same vesicular
structures (probably endosomes) as the transferrin.
Monochains Coupled to a Fluorescent Chemical Penetrate Target
30 Cells
      [0120] Purified monochains were chemically linked to FITC
(fluorescein isothiocyanate), as described in "specific
techniques". VAD12.79 cells grown on slides were incubated
with the FITC-conjugated monochain, transferrin-conjugated-

transferrin or both. The cells were fixed and analyzed by
confocal microscopy (results not shown). The results showed
that both transferrin and the monochain penetrated the cells,
localized in vesicular structures and in fact, a large part of
5   the two molecules was localized to the same organelles,
probably, endosomes.

GFP/Monochain, Penetrates Human Cells

    [0121] In order to follow the trafficking of β2m/HFE
monochain in cells and its internalization by target cells and,
10 in addition, to explore the option of conjugating additional
proteins to the monochain for drug delivery applications, a
GFP-monochain (β2m/HFE/Mg) was engineered. If a β2m/HFE/Mg
monochain penetrates the cells it would imply that other
proteins, such as toxins, regulatory proteins, and antibodies,
15 could be targeted to the endosomes via the β2m/HFE platform.
HeLa and HEK293 cells were transfected with the β2m/HFE/Mg
expression vector. G418 resistant clones were selected and
screened by FACS for the expression of β2m/HFE/Mg monochain
(data not shown). While 90% of the HEK293 clones expressed
20 β2m/HFE/Mg monochain, only 10% of the HeLa clones expressed the
protein. Since the transfection efficiency of both cell lines
was the same, these data further support the previous
assumption that HFE-derivatives are down-regulated in HeLa
cells.

25   [0122] Since HFE is a negative regulator of iron uptake it
was reasoned that its function might be manifested in the
presence of the TfR ligand, the transferrin. Therefore, the
binding and internalization of β2m/HFE/Mg by target cells
either in the absence or presence of human transferrin in the
30 cultured media was tested. HeLa cells grown on cover glass
slides were incubated with conditioned media enriched with
β2m/HFE/Mg protein with or without human transferrin-
conjugated-transferrin. The cells were fixed, washed in PBS
and mounted (results not shown). The β2m/HFE/Mg protein did

not penetrate HeLa cells in media devoid of human transferrin.
However, in the presence of human transferrin, the β2m/HFE/Mg
was internalized by cells and was partially co-localized with
transferrin, probably in the endosomes. The monochain level
5   decreased dramatically following 2 hours incubation, while the
transferrin level was still high. The data clearly
demonstrated that a monochain constructed from three
polypeptides and based on the β2m/HFE core, penetrates cells
via a pathway that depends on TfR/transferrin interaction.

10   [0123] The following conclusions can be drawn from these
results:

    A.   A complex molecule that consists of the soluble
         β2m/HFE and GFP is expressed, properly folded,
         transported via the secretory pathway to the
15   surrounding media and functions as expected in
         mammalian cells. The target cells are able to
         uptake β2m/HFE/Mg for a limited period of time
         suggesting that the amount of transferrin, HFE
         or cell surface TfR limits the uptake.
20   B.   The β2m/HFE/Mg is internalized by target cells
         only in the presence of human transferrin,
         suggesting that the binding of transferrin to
         TfR is essential for high affinity binding of
         β2m/HFE to TfR and/or that internalization of
25   TfR/HFE complexes depends on transferrin-
         binding.
    C.   A portion of the β2m/HFE/Mg molecule is co-
         localized with transferrin, but for a limited
         period of time, suggesting that HFE may
30   dissociate from TfR complex and translocate to
         another cellular organelle.
    D.   The half-life of the β2m/HFE/Mg in the cells is
         relatively short indicating that either it is

degraded or that its fluorescence emission
diminishes with time.

Decreased Level of Cellular TfR in Cells Treated with
β2m/HFE/Mhm

[0124] Over-expression of HFE results in reduction of iron
pools that consequently leads to up-regulation in the level of
TfR and down-regulation in the level of the Fe-storage protein,
ferritin (published data and data not shown). A study was
designed to determine whether external addition of β2m/HFE
monochain leads to the same results. HeLa cells were treated
with purified monochains for 24 hours and TfR levels were
monitored by Western blot analysis. Fig. 9 shows that
treatment of HeLa cells with β2m/HFE, reduced by at least 50%
the level of TfR. Treatment with a Fe-chelating agent (DFO)
had a marginal effect on the levels of TfR. The decrease was
specific to TfR since such a decrease was not observed in the
level of other proteins like MAP kinase. These data, in
addition to the data shown in the previous paragraph (block in
monochain uptake following 2 hours incubation with the
monochain), suggest that β2m/HFE/Mg induces intracellular
degradation of TfR.

[0125] From all of these experiments, the following
conclusions emerge:

        A.   A recombinant DNA fragment encodes a soluble
β2m/HFE monochain in mammalian cells. The
β2m/HFE monochain fold depends on the location
of the His-tag in the vector. A β2m/HFE/Mhm is
properly folded, soluble, stable and rapidly
secreted to the supernatant from transfected
cells.

        B.   β2m/HFE/Mhm binds to TfR intracellularly
independent of its conformation.

        C.   β2m/HFE/Mhm binds to TfR on the surface of tumor
cells.

       D.   β2m/HFE/Mhm penetrates tumor cells via a TfR-
            dependent pathway.

       E.   β2m/HFE/Mhm can be chemically coupled to other
            chemicals (for example, FITC) or synthesized as
5   a more complex, tri-partner protein (for
            example, β2m/HFE/Mg) and keep its
            characteristics. Thus, the latter protein
            conjugates bind and penetrate cells in a TfR-
            dependent pathway.

10   F.   Some tumor cell lines are sensitive to high
            levels of HFE and HFE derivatives (HeLa, PC3),
            suggesting that HFE derivatives can interfere
            with growth.

       G.   Externally added β2m/HFE/Mhm reduces the level
15   of TfR in some tumor cell lines and thus
            interferes with iron metabolism.

[0126] The foregoing description of the specific embodiments
will so fully reveal the general nature of the invention that
others can, by applying current knowledge, readily modify
20 and/or adapt for various applications such specific embodiments
without undue experimentation and without departing from the
generic concept, and, therefore, such adaptations and
modifications should and are intended to be comprehended within
the meaning and range of equivalents of the disclosed
25 embodiments. It is to be understood that the phraseology or
terminology employed herein is for the purpose of description
and not of limitation. The means, materials, and steps for
carrying out various disclosed functions may take a variety of
alternative forms without departing from the invention. Thus
30 the expressions "means to..." and "means for...", or any method
step language, as may be found in the specification above
and/or in the claims below, followed by a functional statement,
are intended to define and cover whatever structural, physical,
chemical or electrical element or structure, or whatever method

step, which may now or in the future exist which carries out
the recited function, whether or not precisely equivalent to
the embodiment or embodiments disclosed in the specification
above, i.e., other means or steps for carrying out the same
5   functions can be used; and it is intended that such expressions
be given their broadest interpretation.

REFERENCES

Ali et al, "Transferrin Trojan horses as a rational approach
    for the biological delivery of therapeutic peptide domain"
    J Biol Chem 274(34):24066-24073 (1999)

Apseloff et al, "Gallium nitrate suppresses lupus in MRL/lpr
    mice", Naunyn-shmiedeberg's Arch Pharmacol 356:517-525
    (1997)

Bahram et al, "Experimental hemochromatosis due to MHC class I
    HFE deficiency: immune status and iron metabolism", Proc
    Natl Acad Sci USA 96(23):13312-13317 (1999)

Barton et al, "Genetic and clinical description of
    hemochromatosis probands and heterozygotes: evidence that
    multiple genes linked to the major histocompatibility
    complex are responsible for hemochromatosis", Blood Cells
    Mol Dis 23(1):135-145 (1997)

Bayer et al, "Differential effects of transferrin receptor
    blockade on the cellular mechanisms involved in graft
    rejection", Transpl Immunol 7:131-139 (1999)

Bennett et al, "Crystal structure of the hereditary
    haemochromatosis protein HFE complexed with transferrin
    receptor", Nature 403:46-53 (2000)

Beutler et al, "Mutation analysis in hereditary
    hemochromatosis", Blood Cells Mol Dis 22: 1871-1894 (1996)

Bickel et al, "Delivery of peptides and proteins through the
    blood-brain barrier", Advanced Drug Delivery Reviews
    46,247-279 (2001)

Borot et al, "Mutations in the MHC class I-like candidate gene
    for hemochromatosis in French patients", Immunogenetics
    45:320-324 (1997)

Brooks et al, "Phase Ia trial of murine immunoglobulin A anti
    transferrin receptor antibody 42/6", Clin Cancer Res
    11:1259-1265 (1995).

Carella et al, "Mutation analysis of the HLA-H gene in Italian
    hemochromatosis patients", Am J Hum Genet 60:828-832
    (1997)

Collery et al, "Inhibitory effects of gallium and tris
    galliumIII on A549 human malignant cell line", Anticancer
    Res 20:955-958 (2000)

Corsi et al, "Overexpression of the hereditary hemochromatosis
    protein, HFE, in HeLa cells induces and iron-deficient
    phenotype", _FEBS Lett_ 460:149-152 (1999)

De Sousa et al, "Iron overload in beta 2-microglobulin-
    deficient mice", _Immunol Lett_ 39(2):105-111 (1994)

Ertel et al, "Increased release of soluble tumor necrosis
    factor receptors into blood during clinical sepsis", _Arch
    Surg_ 129(12):1330-1336; discussion 1336-1337 (1994)

Fan et al, (1998). Myc-epitope tagged proteins detected with
    the 9E10 antibody in immunofluorescence and
    immunoprecipitation assays but not in Western blot
    analysis. Biochem Cell Biol 76,125-128.

Feder et al, "A novel MHC class I-like gene is mutated in
    patients with hereditary hemochromatosis" _Nature Gen_
    13:399-408 (1996)

Feder et al "The Hemochromatosis gene product complexes with
    the transferrin receptor and lowers its affinity for
    ligand binding", _Proc Natl Acad Sci USA_ 95:1472-1477
    (1998)

Feder, J N, "The hereditary hemochromatosis gene (HFE): a MHC
    class I-like gene that functions in the regulation of iron
    homeostasis" _Immunol Res_ 20:175-185 (1999)

Fernandes et al, Characterization of MHC class II-presented
    peptides generated from an antigen targeted to different
    endocytic compartment. _Eur J Immunol_ 30:2333-3243 (2000)

Fromm et al, "MHC class I heavy chain mRNA must exceed a
    threshold level for the reconstitution of cell surface
    expression of class I MHC complexes in cells transformed
    by the highly oncogenic adenovirus 12", _J Biol Chem_
    273:15209-16 (1998)

Gross et al, "Co-trafficking of HFE with transferrin receptor
    implies role in intracellular iron regulation", _J Biol
    Chem_ 273:22068-22074 (1998)

Hagihara et al, "Vascular protection by chloroquine during
    brain tumor therapy with Tf-CRM107", _Cancer Res_ 60:230-234
    (2000)

Hall WA, "Targeted toxin therapy for malignant astrocytoma",
    Neurosurgery 46:544-551 (2000)

Ikuta et al, "Overexpression of hemochromatosis protein, HFE,
    alters transferrin recycling process in human hepatoma
    cells", Biochim Biophys Acta 1496(2-3):221-231 (2000)

Jazwinska et al, "Haemochromatosis and HLA-H" Nat Genet 14:249-
    251 (1996)

Jouanolle et al, "Haemochromatosis and HLA-H" Nat Genet 14:251-
    252 (1996)

Kemp et al, "Effects of anti-transferrin receptor antibodies on
    the growth of neoplastic cells", Pathbiology 60(1):27-32
    (1992)

Kemp JD, "Iron deprivation and cancer: a view beginning with
    studies of monoclonal antibodies against the transferrin
    receptor. Histol Histopathol 12:291-296 (1997)

Kohler et al, "Continuous cultures of fused cells secreting
    antibody of predefined specificity", Nature 256:495-497
    (1975).

Kono et al, "Novel gene delivery system: complexes of fusigenic
    polymer-modified liposomes and lipoplexes", Gene Ther
    8(1):5-12 (2001)

Lebron et al "Crystal structure of the hemochromatosis protein
    HFE and characterization of its interaction with
    transferrin receptor", Cell 93:111-123 (1998)

Lebron et al, "The transferrin receptor binding site on HFE,
    the class I MHC-related protein mutated in hereditary
    hemochromatosis", J Mol Biol 289:1109-1118 (1999)

Lesley et al, "Expression of transferrin receptor on murine
    hematopoietic progenitors", Cellular Immunology 83:14-25
    (1984)

Levy et al, "Genes that modify the hemochromatosis phenotype in
    mice" J Clin Invest 105:1209-1216 (2000)

Li et al, "Brain delivery of biotinylated NGF bounded to an
    avidin-transferrin conjugate", J Nat Toxins 9:73-83 (2000)

Lyass et al, "Correlation of toxicity with pharmacokinetics of
    pegylated lyposomal Doxorubicin (Doxil) in metastatic
    breast carcinoma", Cancer 89:1037-1047 (2000)

Meinkoth et al, "Hybridization of nucleic acids immobilized on
    solid supports", Anal Biochem 138:267-284 (1984)

Niederau et al, "Haemochromatosis and the liver", J Hepatol
    30(Suppl 1):6-11 (1999)

Ogris et al, "PEGylated DNA/transferrin-PEI complexes: reduced
    interaction with blood components, extended circulation in
    blood and potential for systemic gene delivery", Gene Ther
    6:595-605 (1999)

Parkkila et al, "Association of transferrin receptor in human
    placenta with HFE, the protein defective in hereditary
    hemochromatosis" Proc Natl Acad Sci USA 94:13198-13202
    (1997)

Ragheb et al, "Cell-surface expression of lymphocyte activation
    markers in myasthenia gravis", Autoimmunity 31(1):55-66
    (1999)

Riedel et al, "HFE downregulates iron uptake from transferrin
    and induces iron-regulatory protein activity in stably
    cells", Blood 94(4):3915-3921 (1999)

Rotem-Yehudar et al, "Down regulation of peptides transporter
    genes in cell lines transformed with the highly oncogenic
    adenovirus 12", J Exp Med. 180:477-488 (1994)

Rothenberg et al, "beta2 knockout mice develop parenchymal iron
    overload: A putative role for class I genes of the major
    histocompatibility complex in iron metabolism", Proc Natl
    Acad Sci USA 93:1529-1534 (1996)

Roy et al, "The hereditary hemochromatosis protein, HFE,
    specifically regulates transferrin-mediated iron uptake in
    HeLa cells", J Biol Chem. 274:9022-9028 (1999)

Salter-Cid et al, "Transferrin receptor is negatively modulated
    by the hemochromatosis protein HFE: implications for
    cellular iron homeostasis", Proc Natl Acad Sci USA
    96:5434-5439 (1999)

Singh M, "Transferrin as a targeting ligand for liposomes and
    anti cancer drugs", Curr Pharm Des 5:443-451 (1999)

Szilagyi et al, "Purification, characterization and substrate
    specificity of rat pancreatic elastase II", Biochim
    Biophys Acta 1251(1):55-65 (1995)

Tada et al, "A new surface antigen (PC.2) expressed exclusively
    on plasma cells", Immunogenetics 11:351-362 (1980)

Taetle et al, "Effects of monoclonal anti-transferrin receptor
    antibodies on in vitro growth of human solid tumors",
    Cancer Res 47:2040-2044 (1987)

Waheed et al, "Association of HFE protein with transferrin
     receptor in crypt enterocytes of human duodenum", _Proc
     Natl Acad Sci USA_ 96:1579-1584 (1999)

Wang et al, "Doxorubicin-gallium-transferrin conjugate
     overcomes multi drug resistance: evidence for drug
     accumulation in the nucleus of drug resistant MCF-7/ADR
     cells", _Anti Cancer Res_ 20:799-808 (2000)

Webster et al, "A pharmacokinetic and phase III study of
     gallium nitrate in patients with non-small cell lung
     carcinoma", _Cancer Chemother Pharmacol_ 45:55-8 (2000)

Wening et al, "Coupled cellular trafficking and diffusional
     limitations in delivery of immunotoxins to multicell tumor
     spheroids", _Biothechnol Bioeng_ 62:562-75 (1999)

White et al, "Combinations of anti-transferrin receptor
     monoclonal antibodies inhibit human tumor cell growth in
     vitro and in vivo: evidence for synergistic
     antiproliferative effects", _Cancer Res_ 50(19):6295-6301
     (1990)

Worwood, M. (1997). The laboratory assessment of iron status--
     an update", _Clin Chim Acta_ 259(1-2):3-23 (1997)

Yanagihara et al, "Effects of epidermal growth factor,
     transferrin and insulin on lipofection efficiency in human
     lung carcinoma cells", _Cancer Gene Ther_ 7:59-65 (2000)

Zhang et al, "Neuroprotection in transient focal brain ischemia
     after delayed intravenous administration of brain-derived-
     neurotrophic factor conjugated to a blood-brain-barrier
     drug targeting system", _Stroke_ 32(6):1378-1384 (2001)

Zhou et al, "HFE gene knockout produces mouse model of
     hereditary hemochromatosis", _Proc Natl Acad Sci USA_
     95(5):2492-2497 (1998)

WHAT IS CLAIMED IS:

1.   A soluble β2m/HFE monochain which is a polypeptide comprising human β2m, or an analog or active fragment thereof, linked to the α1-α3 domains of human HFE, or an analog or active fragment thereof, by means of a flexible linker peptide, or a functional derivative or salt of said polypeptide.

2.   A monochain in accordance with claim 1, further including a therapeutic bound thereto.

3.   A DNA molecule comprising a nucleotide sequence encoding the monochain of claim 1 or claim 2.

4.   A vector containing the DNA of claim 3

5.   A cell transformed with a vector in accordance with claim 4.

6.   A method of producing a monochain in accordance with claim 1 or claim 2, comprising culturing cells in accordance with claim 5 under conditions such that said monochain is expressed and collecting the monochain so expressed.

7.   A method for reducing intracellular iron absorption, comprising administering to a patient in need of reduction of intracellular iron absorption an effective amount of a monochain in accordance with claim 1.

8.   A method for reducing intracellular iron absorption, comprising administering to a patient in need of reduction of intracellular iron absorption an effective amount of β2m or an analog, active fragment, functional derivative or salt thereof.

9.   A method for reducing intracellular iron absorption, comprising administering to a patient in need of reduction of intracellular iron absorption an effective amount of HFE or an analog, active fragment, functional derivative or salt thereof.

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10.   A method for delivering a therapeutic to cells that over-express the transferrin receptor (TfR), comprising administering a monochain in accordance with claim 2.

11.   A method for delivering a therapeutic across the blood-brain barrier, comprising administering a monochain in accordance with claim 2.

12.   A monochain in accordance with claim 1 or claim 2, wherein said linker is one to five repetitions of GGGGS (SEQ ID NO:15).

13.   A monochain in accordance with any one of claims 1, 2, or 12, further including a protease cleavage site in or adjacent to said linker.

14.   A monochain in accordance with claim 2, further including a protease cleavage site between said therapeutic and the rest of said monochain.

15.   A monochain in accordance with claim 2, wherein said therapeutic is a chemical, protein, peptide, DNA or oligonucleotide conjugated directly to the monochain or indirectly via liposomes or polymer-modified liposomes.

16.   A method for treatment of a brain tumor comprising administering a monochain in accordance with any one of claims 2, 14 or 15, wherein said therapeutic is one which is effective against said brain tumor, whereby said monochain will deliver said therapeutic across the blood-brain barrier.

17.   A method in accordance with claim 10, wherein said cells are activated lymphocytes or leukocytes.

18.   A method in accordance with claim 9, wherein said patient in need of reduction of iron absorption is one having hereditary hemochromatosis, transfusions, thalassemias, hemolytic anemia or chronic infections.

19.   A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a monochain in accordance with any one of claims 1, 2, or 12-15.
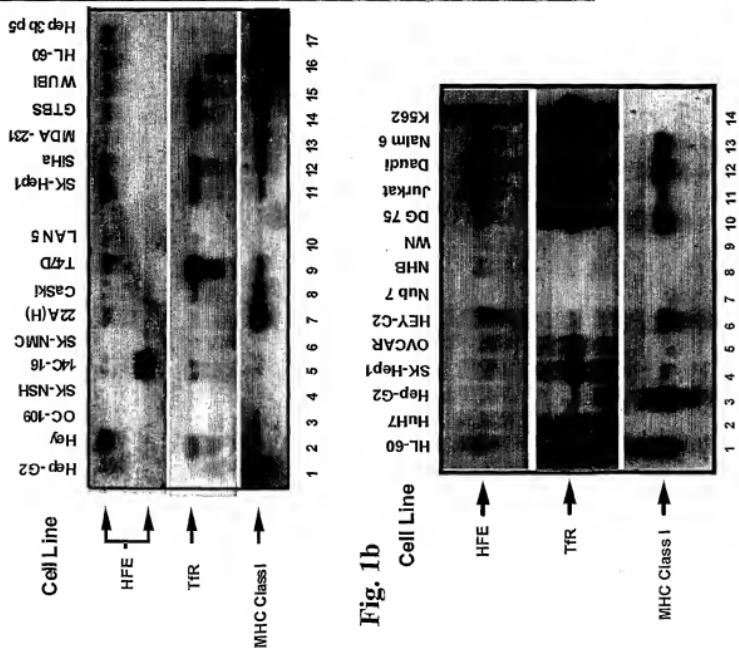
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	SK-NMC	-	-/low	-/low
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	Nub 7	-	-/low	+
	NHB	+	+	-/low
	WN	+	+	++
Hepatoma	Hep-G2	+	++	++
	SK-Hep1	++	++	++
	Hep 3b p5	++	++	++
	HuH7	+	++	++
Cervical Squamous Carcinoma	SiHa	++	++	++
Cervical Epidermal Carcinoma	CaSki	+	+	
Melanoma	GTBS	++	++	++
	WUBI	++	++	++
Ovarian Carcinoma	HEY	+++	+	+
	HEY C2	+	++	+++
	OVCAR	++	++	+
	OC109	-	-/low	+
Breast Carcinoma	T4TD	++	++	++
	MDA 231	+	+/low	+++
Squamous Cell Carcinoma	22A	+	+	++
Burkitt's Lymphoma	14C-16	++	+++	++
	Daudi	+++	+++	+++
Pro B	DG-75	+++	+++	+++
T Cell Leukemia	Nalm 6	+++	+++	+++
Chronic Myelogenous Leukemia	Jurkat	+++	+++	++
	K562	+++	+++	-/low
Promyelocytic Leukemia	HL-60	+++	+++	+++
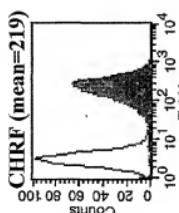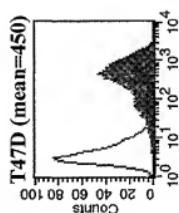
Fig. 1c

Fig. 1a

Fig. 1b

Fig. 1f

Fig. 1i

Fig. 1e

Fig. 1h
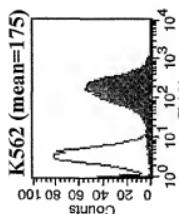
Fig. 1k
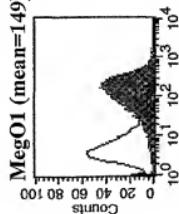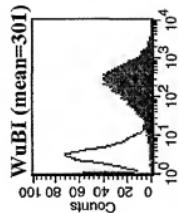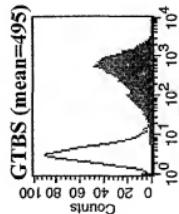
Fig. 1d

Fig. 1g

Fig. 1j

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61/21                                      91/31
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ile gln arg thr pro lys ile gln val tyr ser arg his pro ala glu asn gly lys ser
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asn phe leu asn cys tyr val ser gly phe his pro ser asp ile glu val asp leu leu
181/61                                     211/71
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lys asn gly glu arg ile glu lys val glu his ser asp leu ser phe ser lys asp trp
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ser phe tyr leu leu tyr tyr thr glu phe thr pro thr glu lys asp glu tyr ala cys
301/101                                    331/111
cgt gtg aac cat gtg act ttg tca cag ccc aag ata gtt aag tgg gat cga gac atg gga
arg val asn his val thr leu ser gln pro lys ile val lys trp asp arg asp met gly
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ala leu gly tyr val asp asp gln leu phe val phe tyr asp his glu ser arg arg val
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glu pro arg thr pro trp val ser ser arg ile ser ser gln met trp leu gln leu ser
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661/221                                    691/231
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his asn his ser lys glu ser his thr leu gln val ile leu gly cys glu met gln glu
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asp asn ser thr glu gly tyr trp lys tyr gly tyr asp gly gln asp his leu glu phe
781/261                                    811/271
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leu val lys val thr his his val thr ser ser val thr thr leu arg cys arg ala leu
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```

Fig. 2

```
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1141/381                              1171/391
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thr leu ala val pro pro gly glu glu gln arg tyr thr cys gln val glu his pro gly
1201/401                              1231/411
ctg gat cag ccc ctc att gtg atc tgg ggg ctc gag tct aga ggg ccc ttc gaa caa aaa
leu asp gln pro leu ile val ile trp gly leu glu ser arg gly pro phe glu gln lys
1261/421                              1291/431
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Fig. 2 (continued)

Cell extract

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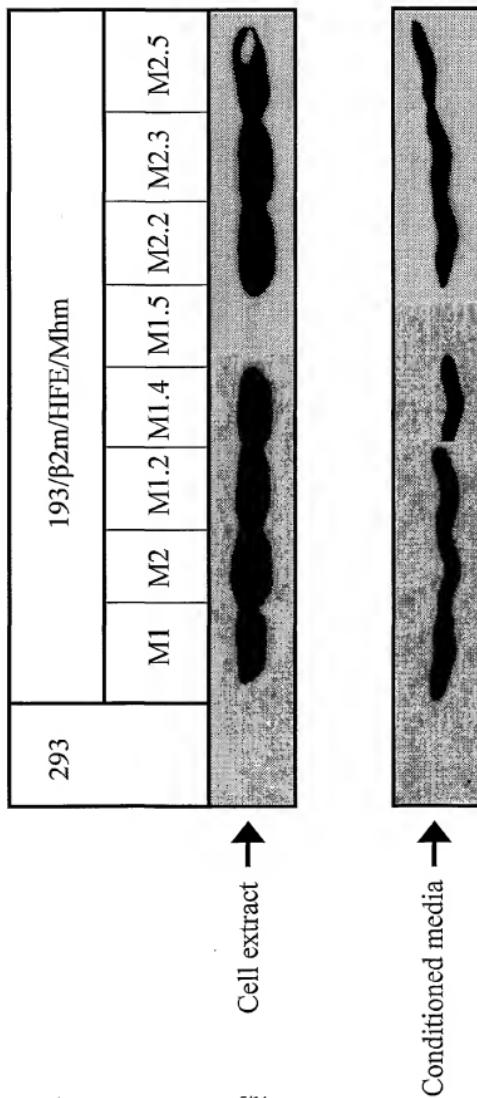
Fig. 3

Fig. 4
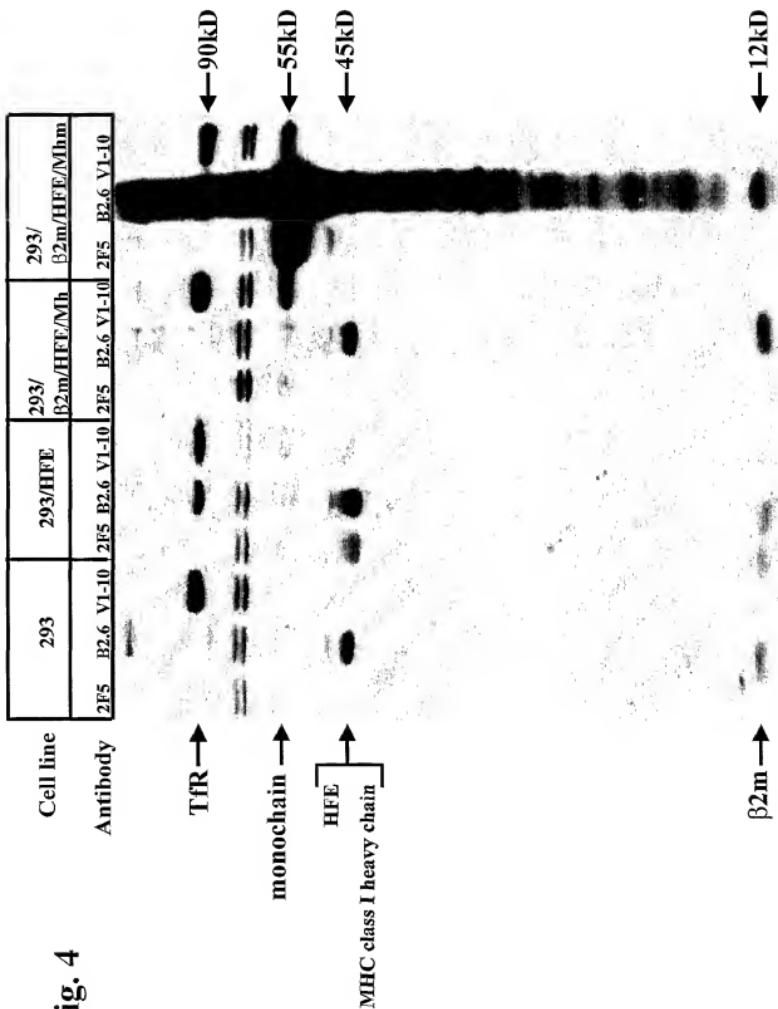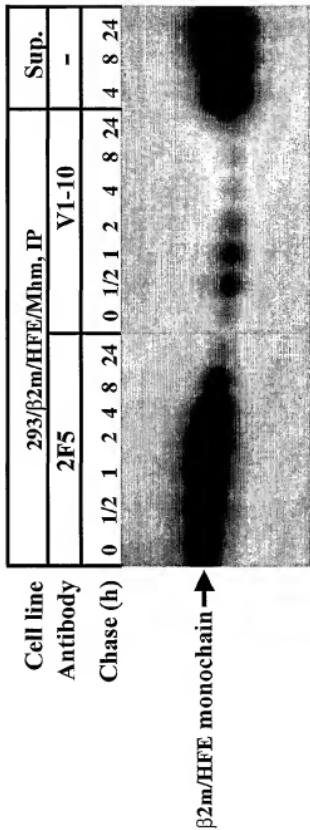
Fig. 5a

Fig. 5b

Fig. 6a

Fig. 6b

Fig. 7a

Cell line	human		mouse		
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Incubation (h)	0 1 24	0 1 24	0 1 24	0 1 24	1 24 0
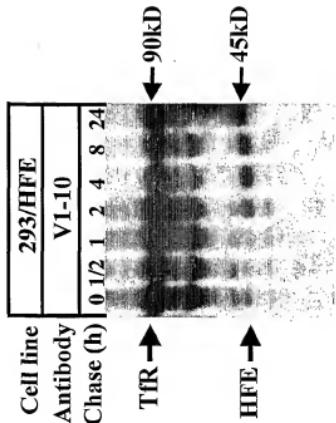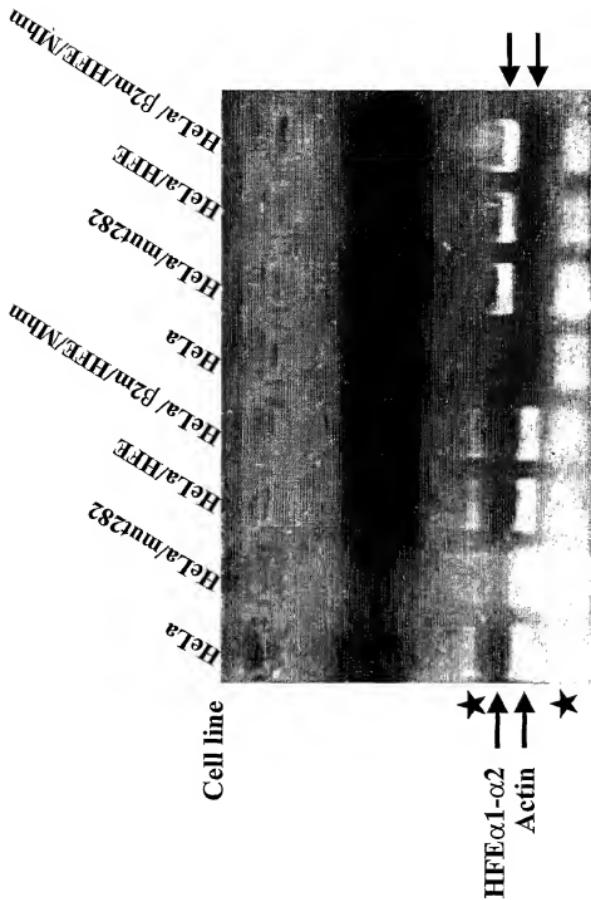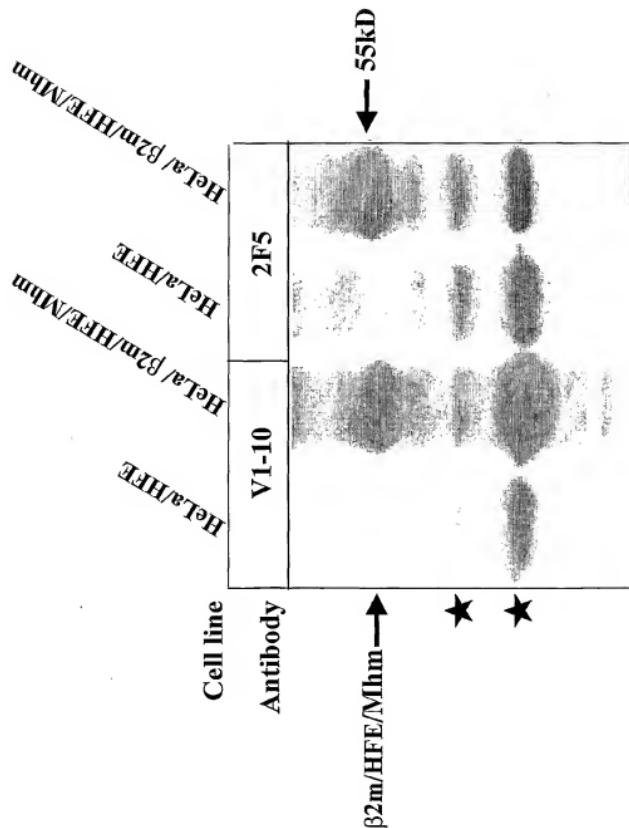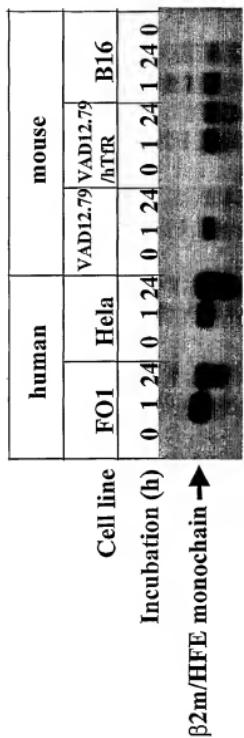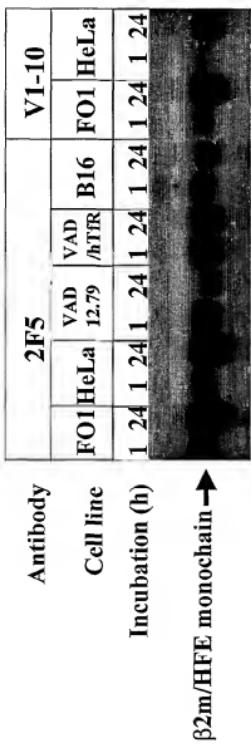
β2m/HFE monochain →

Fig. 7b
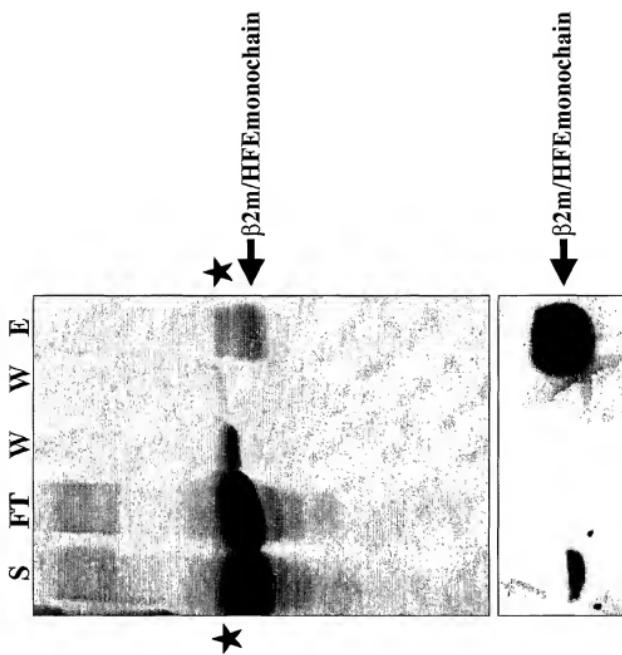
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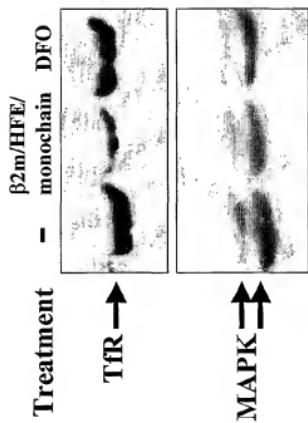
Fig. 9

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       ROTEM-YEHUDAR, Rimat
       LAHAM, Nihay

<120>  A SOLUBLE BETA 2 MICROGLOBULIN (ß2M)/HFE MONOCHAIN FOR BIOTECHNOLOGIC
AL AND
THERAPEUTIC APPLICATIONS

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ggc ctg gag gct atc cag cgt act cca aag att cag gtt tac tca cgt      96
Gly Leu Glu Ala Ile Gln Arg Thr Pro Lys Ile Gln Val Tyr Ser Arg
            20                  25                  30

cat cca gca gag aat gga aag tca aat ttc ctg aat tgc tat gtg tct     144
His Pro Ala Glu Asn Gly Lys Ser Asn Phe Leu Asn Cys Tyr Val Ser
        35                  40                  45

ggg ttt cat cca tcc gac att gaa gtt gac tta ctg aag aat gga gag     192
Gly Phe His Pro Ser Asp Ile Glu Val Asp Leu Leu Lys Asn Gly Glu
    50                  55                  60

aga att gaa aaa gtg gag cat tca gac ttg tct ttc agc aag gac tgg     240
Arg Ile Glu Lys Val Glu His Ser Asp Leu Ser Phe Ser Lys Asp Trp
65                  70                  75                  80

tct ttc tat ctc ttg tac tac act gaa ttc acc ccc act gaa aaa gat     288
Ser Phe Tyr Leu Leu Tyr Tyr Thr Glu Phe Thr Pro Thr Glu Lys Asp
                85                  90                  95

gag tat gcc tgc cgt gtg aac cat gtg act ttg tca cag ccc aag ata     336
Glu Tyr Ala Cys Arg Val Asn His Val Thr Leu Ser Gln Pro Lys Ile
            100                 105                 110
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gtt aag tgg gat cga gac atg gga ggt ggc ggc tcg ggc gga ggt gga       384
Val Lys Trp Asp Arg Asp Met Gly Gly Gly Gly Ser Gly Gly Gly Gly
        115                 120                 125

tcc ggt ggc ggc ggt tcc cgc ttg ctg cgt tca cac tct ctg cac tac       432
Ser Gly Gly Gly Gly Ser Arg Leu Leu Arg Ser His Ser Leu His Tyr
        130                 135                 140

ctc ttc atg ggt gcc tca gag cag gac ctt ggt ctt tcc ttg ttt gaa       480
Leu Phe Met Gly Ala Ser Glu Gln Asp Leu Gly Leu Ser Leu Phe Glu
145                 150                 155                 160

gct ttg ggc tac gtg gat gac cag ctg ttc gtg ttc tat gat cat gag       528
Ala Leu Gly Tyr Val Asp Asp Gln Leu Phe Val Phe Tyr Asp His Glu
                165                 170                 175

agt cgc cgt gtg gag ccc cga act cca tgg gtt tcc agt aga att tca       576
Ser Arg Arg Val Glu Pro Arg Thr Pro Trp Val Ser Ser Arg Ile Ser
            180                 185                 190

agc cag atg tgg ctg cag ctg agt cag agt ctg aaa ggg tgg gat cac       624
Ser Gln Met Trp Leu Gln Leu Ser Gln Ser Leu Lys Gly Trp Asp His
            195                 200                 205

atg ttc act gtt gac ttc tgg act att atg gaa aat cac aac cac agc       672
Met Phe Thr Val Asp Phe Trp Thr Ile Met Glu Asn His Asn His Ser
        210                 215                 220

aag gag tcc cac acc ctg cag gtc atc ctg ggc tgt gaa atg caa gaa       720
Lys Glu Ser His Thr Leu Gln Val Ile Leu Gly Cys Glu Met Gln Glu
225                 230                 235                 240

gac aac agt acc gag ggc tac tgg aag tac ggg tat gat ggg cag gac       768
Asp Asn Ser Thr Glu Gly Tyr Trp Lys Tyr Gly Tyr Asp Gly Gln Asp
                245                 250                 255

cac ctt gaa ttc tgc cct gac aca ctg gat tgg aga gca gca gaa ccc       816
His Leu Glu Phe Cys Pro Asp Thr Leu Asp Trp Arg Ala Ala Glu Pro
            260                 265                 270

agg gcc tgg ccc acc aag ctg gag tgg gaa agg cac aag att cgg gcc       864
Arg Ala Trp Pro Thr Lys Leu Glu Trp Glu Arg His Lys Ile Arg Ala
        275                 280                 285

agg cag aac agg gcc tac ctg gag agg gac tgc cct gca cag ctg cag       912
Arg Gln Asn Arg Ala Tyr Leu Glu Arg Asp Cys Pro Ala Gln Leu Gln
        290                 295                 300

cag ttg ctg gag ctg ggg aga ggt gtt ttg gac caa caa gtg cct cct       960
Gln Leu Leu Glu Leu Gly Arg Gly Val Leu Asp Gln Gln Val Pro Pro
305                 310                 315                 320

ttg gtg aag gtg aca cat cat gtg acc tct tca gtg acc act cta cgg      1008
Leu Val Lys Val Thr His His Val Thr Ser Ser Val Thr Thr Leu Arg
                325                 330                 335

tgt cgg gcc ttg aac tac tac ccc cag aac atc acc atg aag tgg ctg      1056
Cys Arg Ala Leu Asn Tyr Tyr Pro Gln Asn Ile Thr Met Lys Trp Leu
            340                 345                 350
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aag gat aag cag cca atg gat gcc aag gag ttc gaa cct aaa gac gta    1104
Lys Asp Lys Gln Pro Met Asp Ala Lys Glu Phe Glu Pro Lys Asp Val
        355                 360                 365

ttg ccc aat ggg gat ggg acc tac cag ggc tgg ata acc ttg gct gta    1152
Leu Pro Asn Gly Asp Gly Thr Tyr Gln Gly Trp Ile Thr Leu Ala Val
    370                 375                 380

ccc cct ggg gaa gag cag aga tat acg tgc cag gtg gag cac cca ggc    1200
Pro Pro Gly Glu Glu Gln Arg Tyr Thr Cys Gln Val Glu His Pro Gly
385                 390                 395                 400

ctg gat cag ccc ctc att gtg atc tgg ggg ctc gag tct aga ggg ccc    1248
Leu Asp Gln Pro Leu Ile Val Ile Trp Gly Leu Glu Ser Arg Gly Pro
                405                 410                 415

ttc gaa caa aaa ctc atc tca gaa gag gat ctg aat atg cat acc ggt    1296
Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly
            420                 425                 430

cat cat cac cat cac cat tga                                         1317
His His His His His His
            435
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Met Ser Arg Ser Val Ala Leu Ala Val Leu Ala Leu Leu Ser Leu Ser
1               5                   10                  15


Gly Leu Glu Ala Ile Gln Arg Thr Pro Lys Ile Gln Val Tyr Ser Arg
            20                  25                  30


His Pro Ala Glu Asn Gly Lys Ser Asn Phe Leu Asn Cys Tyr Val Ser
        35                  40                  45


Gly Phe His Pro Ser Asp Ile Glu Val Asp Leu Leu Lys Asn Gly Glu
    50                  55                  60


Arg Ile Glu Lys Val Glu His Ser Asp Leu Ser Phe Ser Lys Asp Trp
65                  70                  75                  80


Ser Phe Tyr Leu Leu Tyr Tyr Thr Glu Phe Thr Pro Thr Glu Lys Asp
            85                  90                  95


Glu Tyr Ala Cys Arg Val Asn His Val Thr Leu Ser Gln Pro Lys Ile
            100                 105                 110
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Val Lys Trp Asp Arg Asp Met Gly Gly Gly Gly Ser Gly Gly Gly Gly
        115   120   125

Ser Gly Gly Gly Gly Ser Arg Leu Leu Arg Ser His Ser Leu His Tyr
    130   135   140

Leu Phe Met Gly Ala Ser Glu Gln Asp Leu Gly Leu Ser Leu Phe Glu
145   150   155   160

Ala Leu Gly Tyr Val Asp Asp Gln Leu Phe Val Phe Tyr Asp His Glu
                165   170   175

Ser Arg Arg Val Glu Pro Arg Thr Pro Trp Val Ser Ser Arg Ile Ser
            180   185   190

Ser Gln Met Trp Leu Gln Leu Ser Gln Ser Leu Lys Gly Trp Asp His
        195   200   205

Met Phe Thr Val Asp Phe Trp Thr Ile Met Glu Asn His Asn His Ser
    210   215   220

Lys Glu Ser His Thr Leu Gln Val Ile Leu Gly Cys Glu Met Gln Glu
225   230   235   240

Asp Asn Ser Thr Glu Gly Tyr Trp Lys Tyr Gly Tyr Asp Gly Gln Asp
                245   250   255

His Leu Glu Phe Cys Pro Asp Thr Leu Asp Trp Arg Ala Ala Glu Pro
            260   265   270

Arg Ala Trp Pro Thr Lys Leu Glu Trp Glu Arg His Lys Ile Arg Ala
        275   280   285

Arg Gln Asn Arg Ala Tyr Leu Glu Arg Asp Cys Pro Ala Gln Leu Gln
    290   295   300

Gln Leu Leu Glu Leu Gly Arg Gly Val Leu Asp Gln Gln Val Pro Pro
305   310   315   320

Leu Val Lys Val Thr His His Val Thr Ser Ser Val Thr Thr Leu Arg
                325   330   335

Cys Arg Ala Leu Asn Tyr Tyr Pro Gln Asn Ile Thr Met Lys Trp Leu
            340   345   350

Lys Asp Lys Gln Pro Met Asp Ala Lys Glu Phe Glu Pro Lys Asp Val
         355   360   365

Leu Pro Asn Gly Asp Gly Thr Tyr Gln Gly Trp Ile Thr Leu Ala Val
      370   375   380

Pro Pro Gly Glu Glu Gln Arg Tyr Thr Cys Gln Val Glu His Pro Gly
385   390   395   400

Leu Asp Gln Pro Leu Ile Val Ile Trp Gly Leu Glu Ser Arg Gly Pro
                405   410   415

Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu Asn Met His Thr Gly
            420   425   430

His His His His His His
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1   5   10   15

Ala Glu Arg Glu
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<223> synthetic

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Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1   5   10   15

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ccatctcgag gtcgccacca tggtgag                                         27


<210>  14
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<400>  14
gctctagagg acttgtacag ctcgtcc                                         27


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<220>
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